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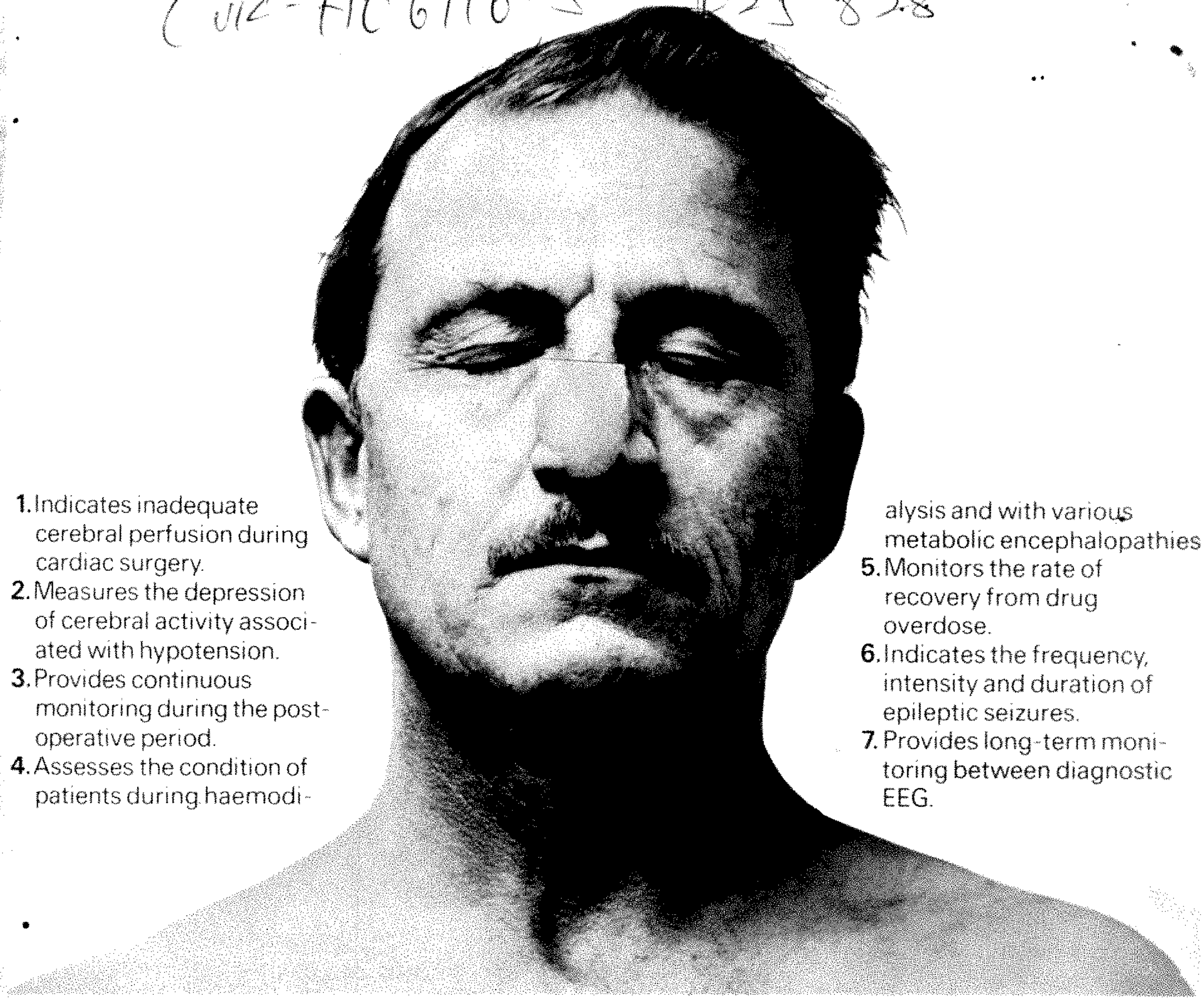

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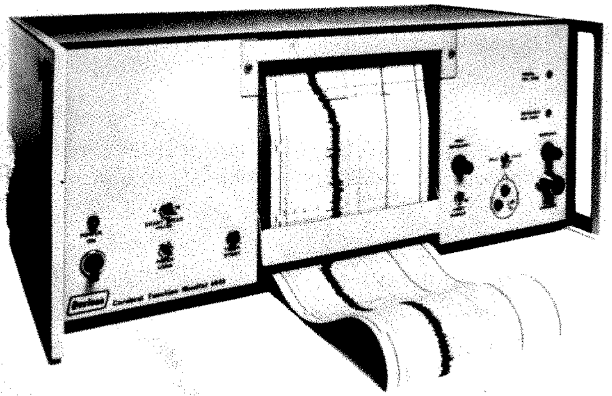

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EFFECTS OF AMINO ACIDS AND CONVULSANTS ON SPONTANEOUS ACTION POTENTIALS IN CEREBELLAR CORTEX SLICES

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1 Picrotoxin selectively and reversibly suppressed the inhibitory action of γ-aminobutyric acid (GABA), but not that of glycine, taurine or β-alanine, on the frequency of spontaneous spike discharges in guinea-pig cerebellar slices. Strychnine reversibly suppressed the inhibitory action of glycine, taurine or β-alanine but had no effect on that of GABA.

2 GABA, glycine, taurine and β-alanine showed an early excitatory effect that was unaffected by picrotoxin or strychnine.

3 Studies of the dose-response relations indicated a competition between the amino acid and the convulsant at a common receptor site.

4 Kinetic analyses of the dose-response relations for the amino acids in the presence or absence of picrotoxin or strychnine indicated that the number of molecules of amino acid combining with the receptor site in order to produce a response (inhibition or excitation) was 3 for GABA, 2 for glycine, 3 for taurine and 4 for β-alanine. There appeared to be no evidence that the response was due to the cooperativity between the amino acid receptor complexes. The number of molecules of convulsant that combined with the receptor site was 1 for either strychnine or picrotoxin.

5 Mixtures of glycine with taurine or β-alanine, in contrast to those with GABA, appeared not to give additive inhibitory effects.

Introduction

It is now known that spontaneous action potentials are exhibited by isolated cerebellar slices of the frog (Hackett, 1972), and of the guinea-pig (Gardner-Medwin, 1972; Okamoto & Quastel, 1973; Yamamoto, 1973) and in cultured rat cerebellum (Hild & Tasaki, 1962; Gähwiler, Mamoon, Schlapfer & Tobias, 1972; Geller & Woodward, 1974). As the discharge frequency of spontaneous action potentials in the cat cerebellum *in situ* (Eccles, Ito & Szentágothai, 1967) is inhibited by iontophoretically applied γ-aminobutyric acid (GABA), glycine, taurine or β-alanine (Kawamura & Provini, 1970), we have carried out experiments to observe the effects of these amino acids on the spontaneous action potentials of guinea-pig cerebellar slices and to throw more light on the nature of the interactions between the amino acids and their respective receptor sites. Moreover, as the inhibitory actions of glycine, taurine or β-alanine on Purkinje cells and other CNS neurones have been reported to be antagonized by strychnine (e.g., Curtis, 1969; Curtis, Duggan, Felix & Johnston, 1970; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Curtis, Duggan & Johnston, 1971), while that of GABA is antagonized by picrotoxin (e.g., Woodward, Hoffer, Siggins & Oliver, 1971; Hill & Simmonds, 1973; Hill, Simmonds & Straughan, 1973), we have also carried out experiments to observe quantitatively the relative effects of strychnine and picrotoxin on the responses of isolated cerebellar slices of the guinea-pig to amino acids. The results and conclusions of these experiments are described in this paper.

Methods

The preparation of cerebellar cortex slices and the methodology for the superfusion have been described previously (Okamoto & Quastel, 1973). Briefly, after the guinea-pig was killed by stunning, the slice was prepared (with a Stadie-Riggs slicer) from the superior vermis by cutting the isolated cerebellum parallel to its uppermost surface. The thickness of the slice was about 0.5 mm and care was taken to compress it as little as possible. The whole operation took about 1–2 minutes. The tissue was then promptly transferred to the superfusion chamber, where the slice was placed on a nylon mesh with its original surface upward. It was superfused with the oxygenated control solution (kept at 37°C) for at least 15 min before the first insertion of the electrode. The level of the superfusion solution was maintained just high enough to cover the whole surface of the slice. The flow of the solution, sucked continuously through a small hole at the bottom of the chamber, created a downward force that kept the slice steady on the mesh.

All solutions, including the control solution and those containing substances to be tested, were applied to the slice by superfusion (flow rate $\simeq$ 1 ml/min) through a glass capillary tip (internal diameter of the tip $\simeq$ 0.3 mm) placed just above the surface of the slice and close ($\simeq$0.5 mm) to the recording glass microelectrode (1–2 MΩ, filled with 2.5 M NaCl). The solution around the electrode was exchanged for another by simply closing and opening the corresponding manifold tubes (see Cooke & Quastel, 1973). This took place within 1–2 seconds.

The control solution (pH = 7.4) consisted of (mM): NaCl 125, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 24 and glucose 11. Amino acids or their salts or antagonists to be tested were dissolved in this solution, the pH being unchanged. For the series of experiments with raised K^+, NaCl was reduced by 25 mM and sucrose was added to solutions containing less than 30 mM K^+ to retain iso-osmosity. The solutions in the reservoirs were always bubbled with 95% O_2 and 5% CO_2 and warmed to 37°C by a water jacket placed near the superfusion tip.

Extracellular action potentials (spikes) were recorded on a Mingograf ink writer and magnetic tape recorder simultaneously. Spike discharge frequencies (spikes/s) were recorded also on the Mingograf after passing through a frequency counter. After the experiment the spike frequencies were counted from the magnetic tape and printed out using a PDP-12 computer programmed for spike frequency counting (Cooke, Okamoto & Quastel, 1973). Values of spike discharge rates (spikes/s), for drawing the dose-response curves and for quantitative assessment of the effects of mixed amino acids, were obtained from the printed values of spike frequencies. The spike frequencies were usually obtained by averaging as many as possible of the steady printed values during various periods, e.g., at the stage of the largest inhibition or at the steady stage when the frequency had partially returned towards the control value.

The cells that displayed steady continuous spontaneous discharges (20–100 spikes/s) of relatively large spikes (about 1 mV or greater) at a depth of 300–350 μm and that consistently responded to the amino acids tested, were selected for the present studies (see Okamoto & Quastel, 1973). The general character of the spontaneous spike discharge did not change significantly for several hours, and the responses of the spike frequency to the amino acids and to the antagonists tested were satisfactorily reproducible during a period of several hours.

In order to avoid possible desensitization due to prolonged exposure to the amino acids, that might cause the flattening of the dose-response curves (Feltz, 1973), the amino acids were only applied at increasing concentrations after a washing by superfusion of the control solution following each application.

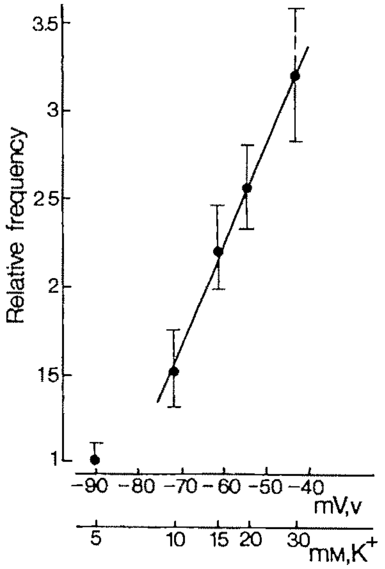

Figure 1 Effects of high-[K^+] on spike discharge frequency. Ordinate scale = frequency relative to that found in 5 mM K^+ solution. Abscissa scale = log [K^+] or calculated membrane potential (v). This was obtained from the Nernst equation, intraneuronal [K^+] being taken as 150 mM. Each point is mean of 3–5 values. Vertical lines show s.e. mean.

Effect of high-K^+ on spike discharge frequency

Experiments were also carried out, for purposes described in the discussion of the kinetic analysis, to examine the relation between the spike frequency and the membrane potential change brought about by the addition of high concentrations of K^+ to the superfusion medium.

It was found that a linear relationship existed between spike frequency and the logarithm of the external [K^+] (10–30 mM). There was an increase of the spike frequency by 5.6%, of the base-line level, for every 1 mV positive shift of the membrane potential calculated from the Nernst equation (Figure 1).

Kinetic analysis

If it is assumed (see Discussion section) that n molecules of amino acid, A, bind stepwise with a receptor, R, and that the response, p, the percentage inhibition (or excitation) of spike discharge frequency by the amino acid, is linearly proportional to the concentration of the complex, A_nR, and also that the concentrations of the intermediate species, AR, ..., $A_{n-1}R$, are negligible compared with those of R and A_nR, then the dose-response curve may be given by eqn. (1) (see Discussion section).

$$p = p_{\max}/[(1 + (K/[A])^n] \qquad (1)$$

where K is the concentration of the amino acid giving 50% of the maximum response ($p_{\max}$). The $p_{\max}$ is 100 for the inhibitory response.

Theoretical curves shown in Figures 4–7 have been calculated from eqn. (1), using various values of n, when no convulsant is present. Such curves, that fitted most closely to the observed values, were those in which the sum of the squares of deviations of the calculated percentage inhibitions from the observed values was minimal.

If m molecules of an antagonist, I, act on R competitively, the dose-response curve in the presence of the antagonist is given by eqn. (2) or (3), assuming that the antagonistic effect is proportional to $[I_mR]$ and that [IR], ..., $[I_{m-1}R]$ are negligible compared with [R] and $[I_mR]$.

$$p' = p_{\max}/\{1 + (K/[A])^n[1 + ([I]/K_I)^m]\} \qquad (2)$$

$$= p_{\max}/[1 + (K'/[A])^n] \qquad (3)$$

where p' = percentage inhibition of spike discharge frequency in the presence of the competitive antagonist, where K′, the concentration of A to give 50% of $p_{\max}$, is equal to $K[1 + ([I]/K_I)^m]^{1/n}$, and where K_I is the apparent dissociation constant $[=(K_{I_1} \cdot K_{I_2} \cdot \ldots \cdot K_{I_m})^{1/m}]$ of the processes: $[I][R] = K_{I_1}[IR]$; $[I][IR] = K_{I_2}[I_2R]$; ....; $[I][I_{m-1}R] = K_{I_m}[I_mR]$.

The value of m may be estimated by eqn. (4) which is derived from the combination of eqns. (1) and (2).

$$\ln[(p-p')/p'] = m\ln([I]/K_I) - \ln[p_{\max}/(p_{\max}-p)] \qquad (4)$$

As $\ln[p_{\max}/(p_{\max}-p)] = \ln[1 + ([A]/K)^n]$ and is therefore constant when [A] is constant, then $\ln[(p-p')/p']$ is linearly proportional to ln [I] and the plot of $\ln[(p-p')/p']$ against ln [I] will give a slope equal to m. The calculated values of m have been obtained in this manner. Examples are shown in Figures 8 and 9.

The values of K_I were then estimated, using the above values of n and m, by means of the equal-response method (Gaddum, 1943; Hubbard, Llinàs & Quastel, 1969) using eqn. (5) which is derived from eqns. (1) and (2) when $p=p'$.

$$K_I = \{[I]^m/[([A_1]/[A_2])^n - 1]\}^{1/m} \qquad (5)$$

where $[A_1]$ and $[A_2]$ are the concentrations of amino acid giving equal response in the presence and absence of the antagonist respectively. The values of n in eqn. (5) are the nearest higher integers to the non-integral n values that give the best fit of the observed data to the theoretical curves (Figures 4–7, see Discussion section).

It is evident from eqn. (5) that the value of $\ln[A_1] - \ln[A_2]$ is a constant depending only on the concentration of the antagonist. Thus, the effect of the antagonist is to shift the entire log dose-response curve to the right, in a parallel manner, when competitive inhibition takes place.

When the binding of I does not exclude binding of A, i.e. for non-competitive inhibition, eqn. (6) holds:

$$p'' = p_{\max}/\{[1 + (K/[A])^n][1 + ([I]/K_I)^m]\} \qquad (6)$$

where p'' = percentage inhibition in the presence of the non-competitive antagonist. Here the effect of the antagonist is to cause a non-parallel shift to the right of the log dose-response curve with a reduction of the maximum response.

Results

Effects of inhibitory amino acids on spike discharge frequencies in cerebellar slices

It has already been shown (Okamoto & Quastel, 1973) that the application, by superfusion, of GABA, glycine or taurine to incubated guinea-pig cerebellar slices greatly reduces the frequencies of spontaneous action potentials. Results of a similar nature have also been obtained with spontaneously firing neurones in cultures of neonatal rat cerebellum (Geller & Woodward, 1974).

The following changes in spike discharge frequencies took place during the superfusion of guinea-pig cerebellar slices with the inhibitory amino acids: (1) There occurred an early excitation followed by inhibition. This was observed with glycine (0.1 to 20 mM), with taurine (0.1 to 10 mM) and with β-alanine (0.2 to 1 mM). The early excitation occurred with GABA at concentrations lower than 0.1 mM but not at 0.2 mM (see Figure 2 or 3). This phenomenon of a phase of excitation followed by inhibition has also been reported by Geller & Woodward (1974) in cultured cells of rat cerebellum. The shortest onset time of the inhibition, i.e., the time between the peak of the early excitation and the time of maximal inhibition was 0.5–2 s with GABA, 1–2 s with β-alanine, 1–5 s with glycine and 5–15 s with taurine (Figure 2 or 3); (2) The onset time and the extent of inhibition were dependent on the amino acid concentration; the higher the concentration the shorter was the onset time and the greater was the extent of inhibition; (3) There was a partial recovery towards the initial frequency of spike discharge (see upward arrows in Figure 2), following the inhibition, although the amino acid was still present in the perfusion solution. The extent of the

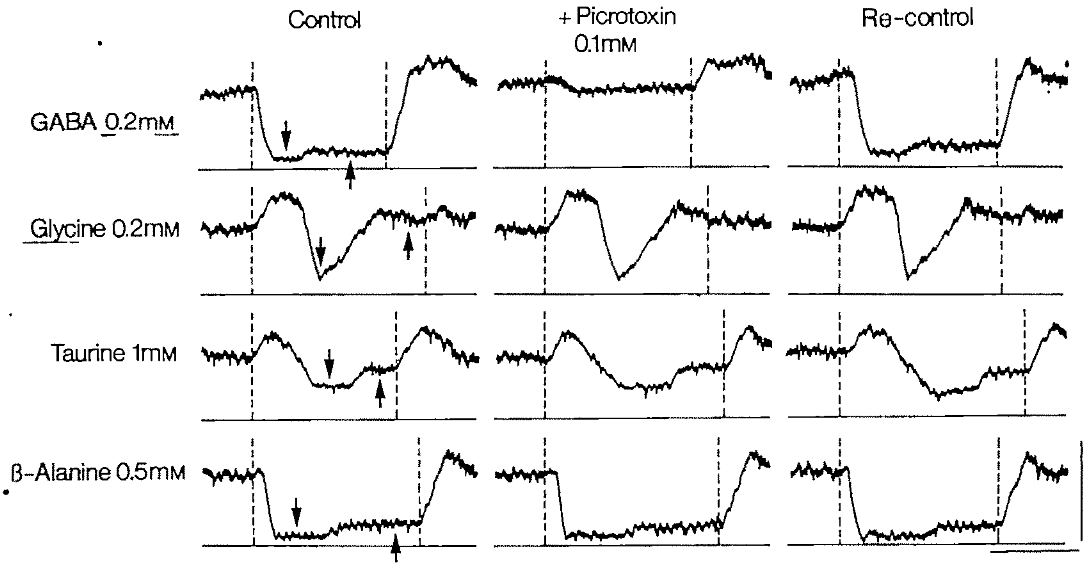

Figure 2 Effects of amino acids and picrotoxin on spike discharge frequencies. All records were obtained from same cell. Amino acids, at concentrations shown, were applied for a period shown by the two vertical broken lines. Picrotoxin (0.1 mM) was applied about 30 s before the application of the amino acids and its effects are given in the middle records. The left and right records are for the control and re-control (picrotoxin-free) conditions respectively. The latter were recorded about 3 min after the removal of the picrotoxin. Scales: vertical = 100 spikes/s; horizontal = 10 seconds. The downward arrows indicate examples of the largest inhibitions produced by the amino acids with which the dose-response plots (Figures 4–7) were obtained. The upward arrows indicate examples of the partially recovered spike frequency in the presence of the amino acids (see Results section).

recovery with GABA, taurine or β-alanine was dependent on the amino acid concentration present in the medium. There was, however, full recovery in the presence of glycine at all concentrations investigated. A similar recovery was reported by Hill *et al.* (1973) using cat cortical neurones *in situ* with microiontophoretic applications of glycine; (4) When the amino acids were removed, by superfusing with the control solution, there was an almost immediate increase of the spike frequency to a value above that of the control, followed by a return in about 10–20 s to the initial discharge rate (Figure 2 or 3). (See also Geller & Woodward, 1974.)

Effects of picrotoxin

Application of picrotoxin, at concentrations lower than 0.1 mM, caused little or no change in the spontaneous spike discharge frequency. Picrotoxin at 1 mM, however, caused an increase of spike discharge frequency that lasted about 20 s, after which the initial rate of spike discharge was resumed.

Application of picrotoxin (0.01–0.1 mM) to the cerebellar slices caused a block of the inhibitory action of GABA (0.05–1 mM, Figure 2). It had no such effect on the inhibitory behaviour of glycine, taurine or β-alanine (Figure 2). Removal of picrotoxin from the superfusion medium resulted, within a few minutes, in the restoration of the original inhibitory activity of GABA. The time of restoration depended on the concentration of picrotoxin used and the length of exposure of the slice to the drug.

The effects of glycine, taurine or β-alanine, on spike discharge rates, at concentrations that gave either just-maximum inhibitions (denoted by downward arrows in Figure 2), or partially-recovered steady states of inhibition (denoted by upward arrows in Figure 2), were unaffected by picrotoxin even at a concentration of 1 mM.

The early excitatory effects on spike discharge frequency exhibited by glycine, taurine or β-alanine were unaffected by picrotoxin (Figure 2), nor was the restoration of spike discharge frequency, on removal of any of these amino acids, affected by picrotoxin (Figure 2).

Effects of strychnine

Strychnine (0.005–0.1 mM) had no noticeable effect on the spike discharge frequency in the absence of

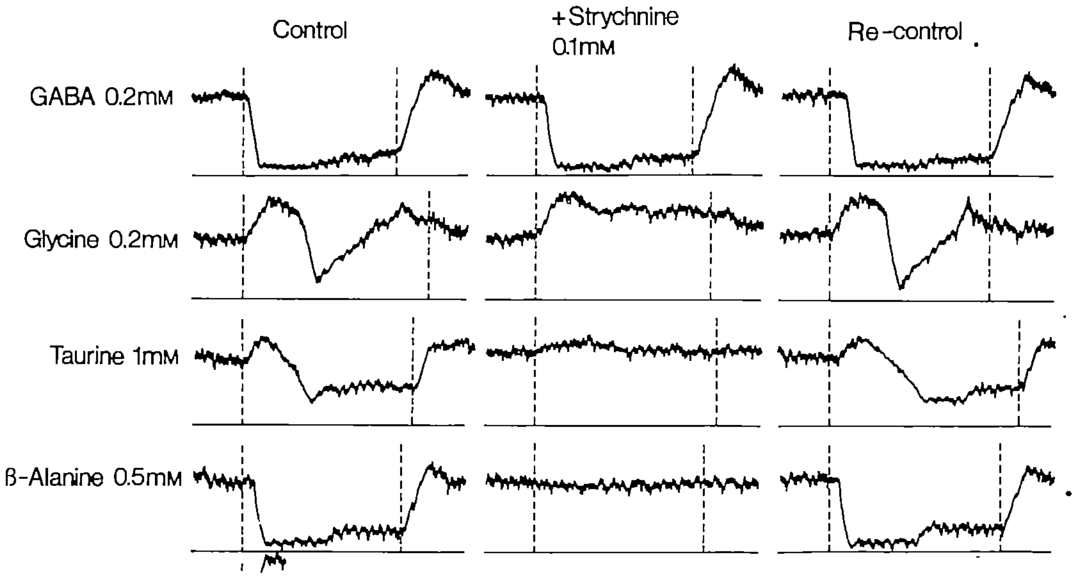

Figure 3 The antagonism by strychnine of inhibitory actions of glycine, taurine and β-alanine. All records were obtained from same cell. The amino acids were applied for a period shown by the two vertical broken lines. Strychnine (0.1 mM), whose effects are given in the middle records, was applied about 30 s before the application of the amino acids. The re-controls, given in the right records, were recorded about 3 min after the removal of strychnine. Scales: vertical = 100 spikes/s; horizontal = 10 seconds.

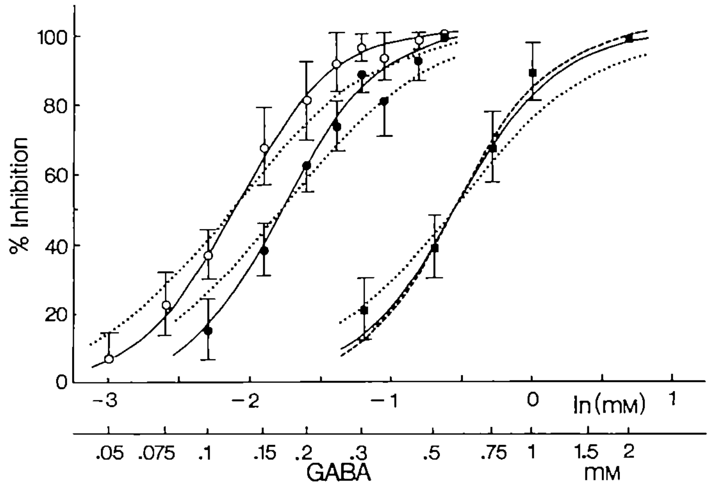

Figure 4 Log dose-response relations of γ-aminobutyric acid (GABA) and the effect of picrotoxin. Ordinate scale = percentage inhibitions of spike discharge frequency by GABA. Abscissa scale = log concentrations (mM) of GABA. Values plotted are means of observed values from 5–8 cells in several slices. Vertical lines show s.e. mean. (○): Control; (●): +10 μM picrotoxin; (■): +100 μM picrotoxin. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to the observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·) and for $n=3$ (– – – –).

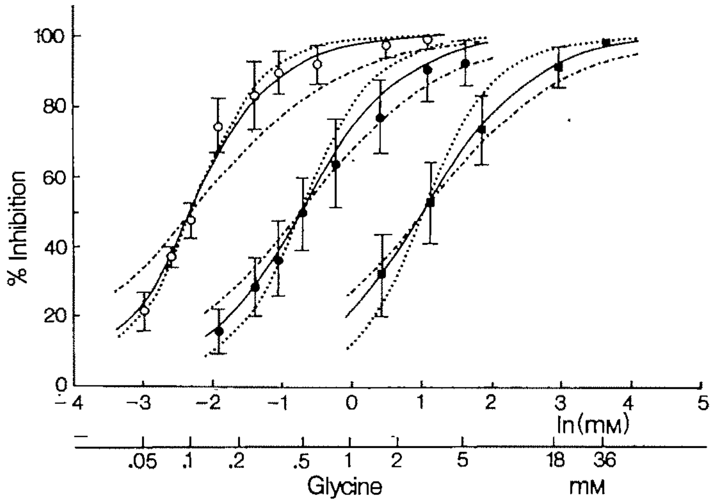

Figure 5 Log dose-response relations of glycine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by glycine. Abscissa scale = log concentrations (mM) of glycine. Values plotted are means of observed values from 5–9 cells in several slices. Vertical lines show s.e. mean. (O): Control; (●): +5 µM strychnine; (■): +50 µM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=1$ (–·–·–·–) and for $n=2$ (······).

added amino acids. Strychnine at high concentrations (e.g. 1 mM) caused a temporary increase of the spike discharge frequency.

The inhibitory action of glycine, taurine or β-alanine on spike discharge frequency was suppressed by strychnine (0.005–0.1 mM) which had no effect on the inhibitory action of GABA (Figure 3). Neither the early excitatory effects of the inhibitory amino acids nor the restoration of spike discharge frequency, on removal of the amino acids, was affected by strychnine (Figure 3). The antagonistic effect of strychnine disappeared within 2 min of removal of the drug (Figure 3).

Dose-response curves of the inhibitory amino acids

The effects of amino acids at different concentrations in the absence and presence of picrotoxin or strychnine on the spike discharge frequencies are shown in Figures 4–7.

The amino acid was applied at different concentrations to a single cell and the percentage inhibitions of the spike frequencies were calculated. The same procedure was repeated with a series of other cells. Averages were then taken of the percentage inhibitions found with a given concentration of this amino acid. As the amino acids under examination exhibited two phases of inhibitory action (see Figure 2, arrows), two types of dose-response curves could be obtained; one type when the largest percentage inhibition of the response was plotted against the dose (Figure 2, downward arrows) and the other when the percentage inhibition, observed when the depressed frequency had recovered to a steady state (Figure 2, upward arrows) was plotted. The first type of curve involved measurements made over a short period (3–5 s), whereas the second type involved measurements made over a period exceeding 12 seconds. Only curves of the first type are shown in Figures 4–7. Curves of the second type were found to be similar to those of the first but are not given because of the anomalous behaviour of glycine and because the reason for the partial recovery with the other amino acids is not yet clear.

Theoretical log dose-response curves are also given in Figures 4–7. They are derived from equation (1) or (3) given in the kinetic analysis using different values of n, the number of amino acid molecules combining at their specific receptor site(s). The values of n that gave the best fit of the theoretical curves to the observed values found in the absence or the presence of the convulsants are shown in Table 1. For the reasons given below the nearest higher integers to the non-integral value of n were assumed to be the correct n values. These were: $n=3$ for GABA, 2 for glycine, 3 for taurine and 4 for β-alanine.

The apparent dissociation constants of the amino acids at their receptor sites, both in the absence and

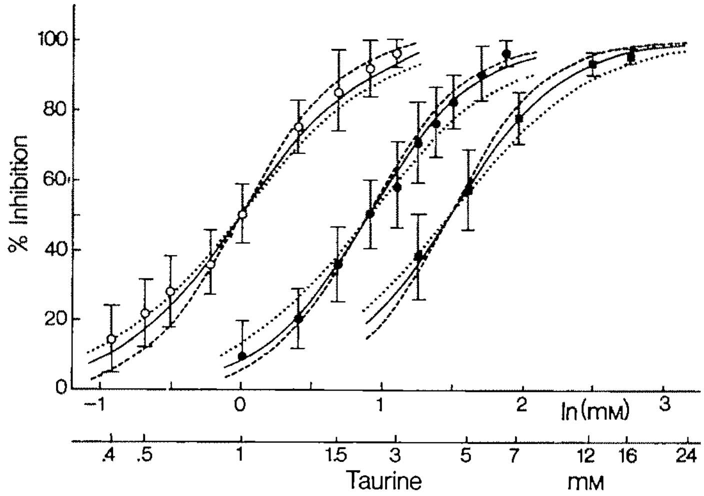

Figure 6 Log dose-response relations of taurine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by taurine. Abscissa scale = log concentrations (mM) of taurine. Values plotted are means of observed values from 7–19 cells in several slices. Vertical lines show s.e. mean. (O): Control; (●): +10 μM strychnine; (■): +50 μM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of *n* for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·) and for $n=3$ (– – – –).

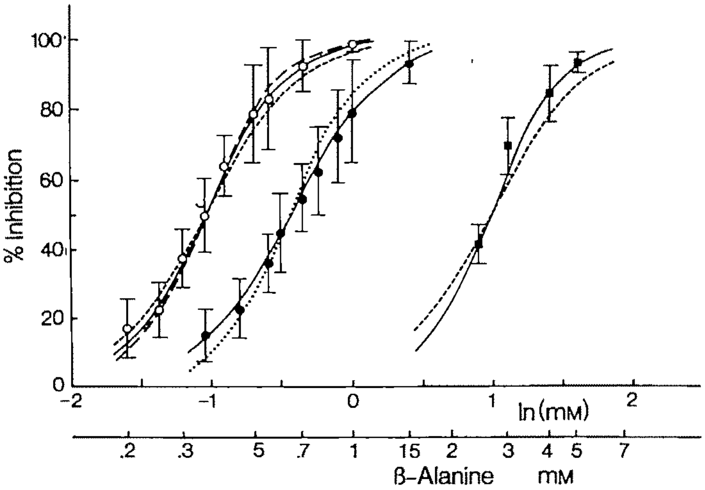

Figure 7 Log dose-response relations of β-alanine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by β-alanine. Abscissa scale ≙ log concentrations (mM) of β-alanine. Values plotted are means of observed values from 5–6 cells in several slices. Vertical lines show s.e. mean. (O): Control; (●): +5 μM strychnine; (■): +50 μM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of *n* for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·), for $n=3$ (– – – –) and for $n=4$ (— — —).

presence of the convulsants, are shown in Table 2. These are derived from equations (1) and (3) in the Kinetic analysis section.

Number (m) of molecules of the convulsant that combine with the receptor site

Picrotoxin brought about a parallel shift to the right of the dose-response curve due to GABA (Figure 4), whilst strychnine brought about a parallel shift to the right of the dose-response curves due to glycine, taurine or βalanine (Figures 5–7). Such parallel shifts by the convulsants indicate a competition between the amino acid and convulsant at a common receptor site (Van Maanen, 1950; Hubbard *et al.*, 1969).

Table 1 Values of *n* giving theoretical dose-response curves having the best fit to the observed values

	n-*values*
GABA	3.0
GABA+picrotoxin (10 µM)	3.0
GABA+picrotoxin (100 µM)	2.8
Glycine	1.7
Glycine+strychnine (5 µM)	1.4
Glycine+strychnine (50 µM)	1.3
Taurine	2.3
Taurine+strychnine (10 µM)	2.6
Taurine+strychnine (50 µM)	2.5
β-Alanine	3.5
β-Alanine+strychnine (5 µM)	3.0
β-Alanine+strychnine (50 µM)	4.0

Values of *n* were calculated from data given in Figures 4–7 using equations (1) and (3) in the absence and presence of convulsants respectively

Values of *m* for picrotoxin and strychnine (eqn. 4) were estimated from the slopes of the linear relations given in Figures 8 and 9 (see Kinetic analysis). The value for *m* for picrotoxin (1–100 µM), in the presence of 0.15 mM GABA, was 1.02 and that for strychnine (1–100 µM) was 0.96, 0.92 and 0.96 in the presence of 1 mM glycine, 3 mM taurine and 1 mM β-alanine respectively.

Linear relationships between the reciprocals of the percentage inhibitions in the presence of constant concentrations of the amino acid and the concentrations of the convulsants added (using the same data as those presented in Figures 8 and 9) were also obtained with either picrotoxin or strychnine. This is to be expected when *m* is equal to 1 (eqn. 4). These results indicated that only one molecule of picrotoxin or strychnine combined with the receptor site. Takeuchi & Takeuchi (1969) have indicated that one molecule of picrotoxin combines with its receptor site in the crayfish neuromuscular junction.

Apparent dissociation constants (K_I) of the convulsant-receptor complexes

The apparent dissociation constants (K_I) of the convulsant-receptor complexes were calculated from eqn. (5) using integral values of *n* (see Kinetic analysis) and with $m = 1$. These values were obtained from the magnitudes of the shifts, at 50% inhibition

Table 2 Apparent kinetic constants of the reactions of amino acids and convulsants

	K or K' (*mM*)	K_I (*µM*)	n *values, for calculation of* K_I
GABA	0.12	—	—
GABA+picrotoxin (10 µM)	0.17	5.1	3
Glycine	0.10	—	—
Glycine+strychnine (5 µM)	0.39	0.25	2
Taurine	1.0	—	—
Taurine+strychnine (10 µM)	2.5	0.35	3
β-Alanine	0.35	—	—
β-Alanine+strychnine (5 µM)	0.65	0.48	4

Values of K or K' and K_I (eqns. 1, 3 and 5), observed from the spike discharge frequency found at the largest depression (denoted by downward arrows in Figure 2), are given. Apparent dissociation constants, K_I, of the convulsant-receptor complexes were calculated from eqn. (5) using the values of the horizontal shifts of the best-fitted log dose-response curves (obtained with a convulsant concentrations of 5 or 10 µM) at 50% inhibition (Figures 4–7). The values of *n* in eqn. (5) used for the calculation of K_I were the nearest higher integers to the non-integral *n* values that gave the best fit of the observed data to the theoretical curves (see solid lines in Figures 4–7).

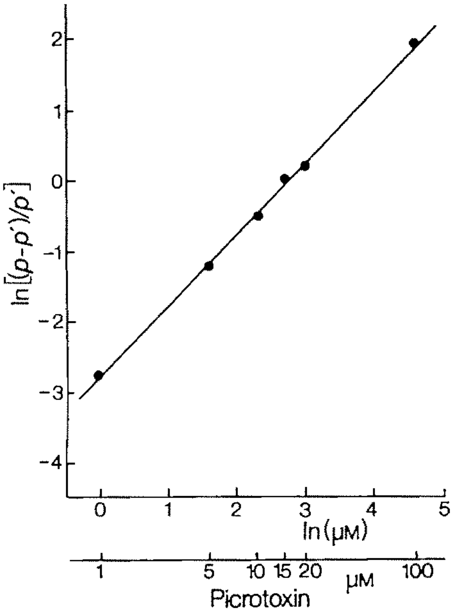

Figure 8 The relation between log[(*p*−*p*′)/*p*′] and log concentrations of picrotoxin in the presence of constant concentration of γ-aminobutyric acid (GABA). Ordinate scale = ln[(*p*−*p*′)/*p*′] where *p* and *p*′ are percentage inhibitions by GABA in the abscence and presence of picrotoxin (eqn. 4). Abscissa scale = ln(μM) of picrotoxin. Values plotted are means of observed values from 4–6 cells in the presence of 0.15 mM GABA. The line, drawn by least mean squares method, gives a slope of 1.0 (see Kinetic analysis, eqn. 4).

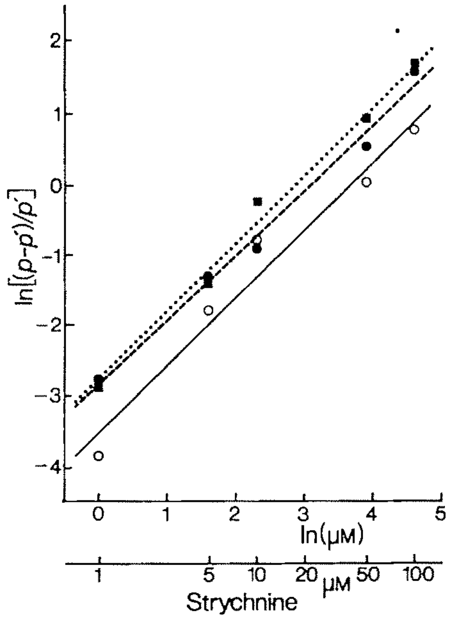

Figure 9 Relations between log[(*p*−*p*′)/*p*′] and log concentrations of strychnine in the presence of constant concentration of glycine, taurine or β-alanine. Ordinate scale = ln[(*p*−*p*′)/*p*′] due to the amino acids in the absence and presence of strychnine. Abscissa scale = ln(μM) of strychnine. Values plotted are means of observed values from 4–6 cells in the presence of 1 mM glycine (■ and dotted line), of 3 mM taurine (● and broken line) and of 1 mM β-alanine (○ and solid line). These lines, drawn by least mean squares method, give slopes of 0.96, 0.92 and 0.96 in the presence of glycine, taurine and β-alanine respectively (see eqn. 4).

brought about by the convulsants (5 or 10 μM), in the best-fitted log dose-response curves shown in Figures 4–7. The values of K_I obtained by this equal response method are given in Table 2.

Interrelations between the inhibitory amino acids

The possibility that glycine, taurine and β-alanine, whose inhibitory activities are similarly affected by strychnine, act at the same receptor site was examined. This was done by carrying out experiments with mixtures of the inhibitory amino acids.

Examples of the effects of mixing inhibitory amino acids on the spike discharge frequencies are shown in Table 3. With mixtures of GABA and glycine, taurine or β-alanine the net inhibition was equal to or slightly greater than the sum of the inhibitions produced by any of the amino acids alone at the concentrations tested. The same was true for a mixture of taurine and β-alanine. However, with mixtures of glycine and either taurine or β-alanine the net inhibition was less than the sum of inhibitions produced by the amino acids, i.e., the percentage inhibition by taurine or β-alanine was reduced in the presence of glycine. Data in Table 3 show the net inhibitions measured at the peaks of the inhibitory action. They indicated an affinity of glycine for receptors of taurine and β-alanine.

Discussion

Kinetic analyses of dose-response relations of amino acids and convulsants

Two assumptions were made in the analysis of the log dose-response curves of the amino acids in the presence and absence of the convulsants. The first assumption was that the spike discharge frequency was directly proportional to the occupancy of the receptor site, i.e., to the amounts of the amino acid-receptor complex, A_nR (see Kinetic analysis). The second assumption was that the amounts of the intermediate amino acid-receptor complexes, AR, . . ., $A_{n-1}R$. (see Kinetic analysis) were negligible compared with those of R and A_nR.

The first assumption may be justified by the following considerations: the interaction between an amino acid, A, and a receptor, R, may be expressed by eqn. (7) (see e.g. Werman, 1969).

$$[A_nR]/R_t = 1/[1+(K/[A])^n] \qquad (7)$$

where R_t is the total number of receptors.

The membrane conductance change, Δg, is linearly related to the fraction of receptors occupied as given by eqn. (8) (see Werman, 1969).

$$\Delta g = \alpha([A_nR]/R_t) \qquad (8)$$

where α is a proportionality constant.

The change of the membrane potential, v, caused by the membrane conductance change, Δg, is given by eqn. (9) (see Martin, 1955 and Hubbard *et al.*, 1969).

$$v = (E \,.\, \Delta g)/(G + \Delta g) \qquad (9)$$

where E is the difference between the resting membrane potential and the equilibrium potential of the ion concerned, and G is the resting membrane conductance.

On rearrangement of eqn. (9) by use of eqns. (7) and (8) we obtain eqn. (10).

$$v = [(\alpha \,.\, E)/(\alpha + G)]/\{1 + [G/(\alpha + G)](K/[A])^n\}$$
$$= \beta/[1 + \gamma(K/[A])^n] \qquad (10)$$

where $\beta = (\alpha \,.\, E)/(\alpha + G)$ and $\gamma = G/(\alpha + G)$. Both β and γ are constants. The relation between v and [A] in eqn. (10) is similar to that between *p*, the spike frequency, and [A] in eqn. (1). As we have shown that a linear relation exists between spike frequencies and the membrane potentials due to varied external high-$[K^+]$ (Figure 1), it may be inferred that the spike frequency is also linearly proportional to the receptor occupancy.

Table 3 Effects of combinations of the amino acids on spike discharge frequency.

	% Inhibition		
GABA (mM)	*+Nil*	*+Glycine (0.05 mM)*	*+Taurine (1 mM)*
0	0	21 ± 6 (7)	50 ± 9 (17)
0.075	22 ± 10 (7)	45 ± 9 (5)	79 ± 7 (5)
0.1	37 ± 7 (8)	64 ± 7 (5)	93 ± 10 (4)
0.15	67 ± 11 (7)	86 ± 7 (5)	100 ± 0 (4)

	% Inhibition			
β-Alanine (mM)	*+Nil*	*+GABA (0.1 mM)*	*+Glycine (0.1 mM)*	*+Taurine (1 mM)*
0	0	37 ± 7 (8)	48 ± 5 (7)	50 ± 9 (17)
0.2	17 ± 10 (5)	56 ± 6 (6)	38 ± 8 (6)	69 ± 8 (5)
0.25	22 ± 9 (5)	71 ± 5 (7)	38 ± 11 (5)	78 ± 6 (5)
0.35	50 ± 10 (6)	—	52 ± 11 (5)	100 ± 0 (4)
0.4	64 ± 8 (5)	—	—	—

	% Inhibition	
Taurine (mM)	*+Nil*	*+Glycine (0.1 mM)*
0	0	48 ± 5 (7)
0.5	22 ± 18 (5)	45 ± 13 (5)
1	50 ± 9 (17)	53 ± 7 (6)
1.5	75 ± 8 (6)	63 ± 10 (5)

Values of percentage inhibitions (means ± s.e. mean) of spike discharge frequencies obtained at the stage of the largest inhibition, as denoted by downward arrows in Figure 2, are given. Number of the cells examined are shown in parentheses.

On the basis of the second assumption non-integral values of n (derived from eqn. (1) or (3)) were found to give curves that gave the best fit to the experimental results (Figures 4–7). As the true n value should be larger than the non-integral values (Cooke *et al.*, 1973) the nearest higher integers to the calculated non-integral values were considered to be the true n values (see Table 2).

It is evident, from eqn. (1), that the ratio $(d \log p)/(d \log [A])$ should approach the value of n with decrease in concentration of A, as indicated by eqn. (11).

$$(d \log p)/(d \log [A]) = n/[1 + ([A]/K)^n] \quad (11)$$

The values of n given by this ratio are recorded in Table 4. There is no significant difference between the values of n given in Table 4, obtained from observations of the ratio $(d \log p)/(d \log [A])$ at low concentrations of A, and those given in Table 1, obtained from the best fits of the theoretical curves to the experimental values observed over a whole range of concentrations of A.

The ratios, $(d \log p)/(d \log [A])$, shown in Table 4 calculated from the values obtained from several individual cells (Table 4, column A), and those calculated from the averaged values obtained from a group of cells (Table 4, column B) are not significantly different. It is, therefore, reasonable to infer that the log dose-response curves shown in Figures 4–7 will represent the behaviour of an individual cell as well as that of a group of cells.

Table 4 Values of the ratio $(d \log p)/(d \log [A])$

	(d log p)/(d log [A])	
	A	*B*
GABA	2.9 ± 0.3 (6)	2.8 (6)
Glycine	1.2 ± 0.1 (5)	1.2 (5)
Taurine	2.5 ± 0.2 (5)	2.1 (8)
β-Alanine	3.4 ± 0.3 (5)	3.1 (6)

Values of $(d \log p)/(d \log [A])$ (see eqn. 11) were calculated from percentage inhibitions (p, given in Figures 4–7, in the absence of the convulsants, and in Table 3) due to the amino acid at two different concentrations of amino acids, corresponding to the lower portions of the log dose-response curves (Figures 4–7). The concentrations used were 0.05 mM and 0.075 mM for γ-aminobutyric acid (GABA); 0.05 mM and 0.1 mM for glycine; 0.4 mM and 0.5 mM for taurine, and 0.25 mM and 0.3 mM for β-alanine. The values shown were calculated in the following manner; e.g. $p = 22\%$ and 7% with 0.075 mM and 0.05 mM GABA respectively, therefore $(d \log p)/(d \log [A]) = (\Delta \log p)/(\Delta \log [A]) = \log (22/7)/\log (0.075/0.05) = 2.8$. Column A shows means (± s.e. mean) of the values calculated from values of p obtained from each of several separate cells. Column B shows values obtained from the averaged p values obtained from a group of cells. The number of cells tested are given in parentheses.

So far as convulsant binding is concerned only one molecule of picrotoxin or strychnine was found to combine with the amino acid receptor site (Figures 8 and 9). A Hill coefficient equal to 1 with strychnine has also been reported by Young & Snyder (1974) using suspensions of a brain fraction.

The parallel shift to the right of the entire log dose-response curves due to the convulsants (Figures 4–7) would indicate an apparently competitive inhibition by strychnine or picrotoxin (see Kinetic analysis). Curtis *et al.* (1971) have suggested that strychnine antagonism to glycine, with cat spinal neurones *in situ*, may be competitive, but Davidoff, Aprison & Werman (1969) have concluded that the antagonism between strychnine and glycine with spike discharges in cat spinal interneurones *in situ* is non-competitive. Takeuchi & Takeuchi (1969) have also reported that the antagonism between GABA and picrotoxin, in their studies of conductance changes, is non-competitive.

So far as combinations of amino acids and convulsants with receptor sites are concerned, Werman (1969) has given the value of n for glycine, with cat spinal interneurones, as at least 2. The value found by our results and listed in Table 2, gives $n = 2$. The number of GABA molecules combining with its receptor site has been reported to be 3 with crayfish muscle (Feltz, 1973) and with locust muscle (Werman & Brookes, 1969). The value for GABA obtained in our studies (Table 2) is $n = 3$. The concentration of GABA needed to give 50% of the maximum conductance increase in crayfish muscle has been quoted as 80 μM (Feltz, 1973) which may be compared with the value 120 μM given in Table 2. The dissociation constant for picrotoxin with crayfish muscle has been reported as 3.3 μM (Takeuchi & Takeuchi, 1969) which is about the same order as the value 5.1 μM that we have obtained (Table 2).

Co-operativity

It is possible to consider the response by inhibitory amino acids to be due either to a combination of the receptor (R) with n molecules of amino acid (A) to give A_nR whose amount determines the magnitude of the responses, as described in Kinetic analysis, or due to the co-operation of n molecules of AR, the complex A_nR_n controlling the amount of response. Under the latter circumstance the following equations apply:

$$A + R \rightleftharpoons AR \quad \text{where} \quad K_1 = [A] \cdot [R]/[AR],$$

$$n \cdot AR \rightleftharpoons A_nR_n \quad \text{where} \quad K_n = [AR]^n/[A_nR_n],$$

and

$$R_t = [R] + [AR] = [AR] \cdot [1 + (K_1/[A])]$$

where R_t is the total number of receptor sites. Therefore, $[AR] = R_t/[1 + (K_1/[A])]$. Then p, the percentage inhibition of spike frequency, is proportional to $[A_nR_n]$ and therefore:

$$\begin{aligned} p &= C \cdot [A_nR_n] = C \cdot [AR]^n/K_n \\ &= [[C \cdot (R_t)^n]/K_n]/[1 + (K_1/[A])]^n \\ &= p_{max}/[1 + (K_1/[A])]^n \qquad (12) \end{aligned}$$

where C is a proportionality constant and p_{max} is the maximum percentage inhibition (equal to 100) obtained when [A] is large (see also Werman, 1969).

It is possible to decide whether equation (1) or eqn. (12) gives constant values of K or K_1 for various experimental values of p obtained with different amino acid concentrations. For non-co-operativity (eqn. 1), $p = 100/[1 + (K/[A])^n]$, i.e., $K = [A][(100/p) - 1]^{1/n}$; and for co-operativity (eqn. 12), $p = 100/[1 + (K_1/[A])]^n$, i.e., $K_1 = [A][(100/p)^{1/n} - 1]$. Values of p at different [A] used for the calculations of K or K_1 are taken from Figures 4–7.

Relevant values of K or K_1 for the response due to GABA are shown in Table 5. It is evident that constancy of K or K_1 only occurs when $n = 3$ for non-co-operativity. Similar results are obtained with glycine ($n = 2$), taurine ($n = 3$) and β-alanine ($n = 4$).

We infer, therefore, that co-operativity, in the sense used in this discussion, is not essential for an interpretation of the kinetics of the responses by inhibitory amino acids.

Receptor sites for the amino acids

It is evident that the receptor site for GABA must differ from that (or those) for glycine, taurine or β-alanine because of the specific antagonistic effects of picrotoxin and strychnine (Figures 4–7). The additive inhibitory effects observed with mixtures of GABA and taurine, glycine or β-alanine (Table 3) also suggest the existence of separate sites for GABA and the other amino acids. Additive inhibitory effects were also observed with mixtures of taurine and β-alanine (Table 3). More data are required, however, before it can be concluded that there are separate receptor sites for taurine and β-alanine, and possibly for glycine.

It seems to be clear that glycine affects the response to β-alanine or taurine (or *vice versa*) but not to GABA. It should be noted that in contrast to the other three inhibitory amino acids, the inhibitory action of glycine on spike frequency tends to disappear and the original frequency is quickly restored (Figure 2 or 3) (see also Hill *et al.,* 1973). This may indicate a possible desensitization by glycine of the receptor for glycine itself or that for taurine or β-alanine, leading to smaller inhibitory effects of taurine or β-alanine in the presence of glycine (Table 3).

We gratefully acknowledge financial assistance of the Medical Research Council of Canada and thank the Department of Pharmacology, University of British Columbia, for laboratory facilities for this work.

Table 5 Values of K or K_1 for response due to γ-aminobutyric acid (GABA) estimated from eqn. (12), based on co-operativity, and from eqn. (1), based on non-co-operativity

GABA [A] (mM)	n	$K_1 = [A][(100/p)^{1/n} - 1]$ *for co-operativity (eqn. (12))*	$K = [A][100/p) - 1]^{1/n}$ *for non-co-operativity (eqn. (1))*
0.1	2	0.065	0.132
0.15	2	0.033	0.105
0.2	2	0.023	0.100
0.25	2	0.012	0.078
0.1	3	0.040	0.120
0.15	3	0.021	0.119
0.2	3	0.015	0.126
0.25	3	0.009	0.115
0.1	4	0.029	0.115
0.15	4	0.016	0.126
0.2	4	0.011	0.141
0.25	4	0.006	0.140

Values of [A] and p are taken from Figure 4.

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A CHOLINOCEPTOR ANTISERUM: ITS PHARMACOLOGICAL PROPERTIES

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1 Sera of rabbits immunized against a nicotinic receptor-rich fraction, obtained from the electric organ of *Torpedo marmorata*, were tested for their pharmacological activity on different *in vitro* preparations.

2 Sera containing antibodies against the nicotinic receptor blocked neuromuscular transmission in the phrenic-nerve hemidiaphragm preparation without affecting the muscle responses evoked by direct electrical stimulation. Complement inactivated sera were still active. Immune sera, incubated for 15 min with a receptor-rich fraction, lost their activity.

3 The immune sera antagonized the responses elicited by acetylcholine on the frog rectus abdominis.

4 The immune sera tested *in vitro* decreased the compound action potential evoked in the superior cervical ganglion of the rat by electrical stimulation of the preganglionic nerve.

5 The sera did not show any activity on muscarinic receptors of the guinea-pig ileum preparation.

6 It is concluded that in sera obtained from immunized rabbits a substance is present with curare-like action, and that this activity is probably due to the presence of antibodies against the nicotinic receptor.

Introduction

It has been demonstrated by Patrick & Lindstrom (1973) that repeated injections of purified *Torpedo* acetylcholine receptors into rabbits induce a flaccid paralysis which has some similarities to human myasthenia gravis. It was assumed that the development of this flaccid paralysis might be due to the production of antibodies against the cholinoceptor protein, and that such antibodies could partially inactivate the rabbit's own cholinoceptors. This experimental model may represent a valid approach in clarifying the pathogenesis of human myasthenia gravis and may support the hypothesis that in this illness the impairment of neuromuscular transmission, and probably of the chemoreceptor of the muscle end-plate (Johns, Grob & Harvey, 1965; Grob, Namba & Feldman, 1966) may be due to an immunological disturbance (Nastuck & Plescia, 1966; Feltkamp, Van Den Berg-Loonen, Nijenhuis, Engelfriet, Van Rossum, Van Loghem & Oosterhuis, 1974). This is consistent with the presence of neuromuscular blocking agents in the sera of myasthenic patients (Berg, 1953; Nastuck, Strauss & Osserman, 1959; Walker, 1973; Laurent, 1973), with the recent findings that have demonstrated a decreased number of active receptor-sites in myasthenic muscles (Fambrough, Drachman & Satyamurti, 1973) and with the presence in myasthenic subjects of a serum globulin which inhibits the binding of α-bungarotoxin to acetylcholine receptors (Alman, Andrew & Appel, 1974). Recently we have produced in rabbits a muscle relaxation that was longer lasting than that previously obtained, and with more similarities to human myasthenia gravis, by immunizing the animals with a cholinoceptor-rich fraction isolated from *Torpedo* electric organs (Berti, Clementi, Conti-Tronconi & Omini, 1974). The results described in this paper demonstrate that a factor which is able to block the nicotinic receptor *in vitro* in a variety of tissues, is present in the serum of such immunized animals.

Methods

Specimens of *Torpedo marmorata* were kindly supplied by Dr Martin, Zoological Station, Naples, and kept in artificial oxygenated sea water at 18–20°C. The electric organs were fractionated according to Cohen, Weber, Huchet & Changeux (1972) as reported in detail elsewhere (Clementi, Conti-Tronconi, Berti & Folco, 1976.

One ml of cholinoceptor-rich fraction containing 1 mg protein was emulsified with 1 ml of complete Freund adjuvant and injected in 10 spots on the back of male albino rabbits (3.0–3.5 kg body weight). The injection was repeated 15 days later and a third dose was given to those animals which did not show muscle weakness. Sterile 0.9% w/v NaCl solution (saline) (40 ml) containing 5% glucose was given subcutaneously, daily, to the treated animals.

Blood was obtained from rabbits by cardiac puncture without anticoagulant 7–10 days from the development of muscle weakness. The blood was allowed to clot at room temperature for 30 min and the serum was separated from blood cells by centrifugation and then stored in a deep freeze. Only sera that gave clear precipitation lines between the undiluted serum and the solubilized receptor protein on immunoassay (Clementi *et al.*, 1975) were used for pharmacological analysis.

Normal and immune sera, diluted in appropriate salt solution, were assayed for their pharmacological activities on a series of *in vitro* preparations. Complement was inactivated by heating the sera at 56°C for 30 minutes.

Rat isolated phrenic nerve-diaphragm

The left hemidiaphragm of male albino rats (Wistar, 180–200 g body weight) was suspended in a 50 ml bath containing Krebs solution according to Bülbring (1946) and bubbled with 95% O_2 and 5% CO_2. The phrenic nerve was stimulated by means of a Grass stimulator with rectangular pulses of 1 ms duration at a frequency of 0.1 Hz and three times the strength necessary to produce a maximal twitch. Direct stimulation was applied between two silver pins embedded in the muscle near its origin in the ribs. Twitches were elicited by stimulation at a frequency of 0.1 Hz, 1 ms duration, and of a strength to produce contractions equal in amplitude to maximal twitches produced by indirect stimulation. The contractions of the muscle were recorded with an isometric transducer.

Frog rectus abdominis

Pithed frogs (*Rana pipiens*) weighing 20–40 g were used and the rectus abdominis muscle was isolated and suspended in a 10 ml bath as described by Van Maanen (1950). The contractions of the muscle were recorded with an isometric transducer.

Rat isolated superior cervical ganglion

Male Wistar rats (70–80 g body weight) were anaesthetized with ethyl-urethane and their right superior cervical ganglion was carefully dissected free. The ganglia, peeled from their connective capsule, were placed, according to Perri, Sacchi & Casella (1970) in a perspex chamber filled with a continuously flowing Krebs solution at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The ganglion cells were excited orthodromically by stimulation of the sympathetic trunk, with silver/silver chloride electrodes. Supramaximal square wave pulses of 0.3 ms duration from a Tektronix generator were applied, via an isolation unit, to the stimulating electrode at frequencies ranging from 1 to 10 Hz. The potentials derived from the internal carotid nerve were led directly to a Tektronix R 5103 N oscilloscope and recorded by a Grass Kymograph camera model C 4PR.

Guinea-pig ileum

Segments of terminal ileum from male guinea-pigs (350–500 g body weight) were placed in a 10 ml organ bath containing Krebs solution gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Tonus and contractions of the organ were recorded by an isometric transducer.

Results

Effects of the immune serum on neuromuscular junction

The normal rabbit serum, with and without complement, at concentrations of up to 2% in the bath had no effect on the electrically evoked contractions of the rat hemidiaphragm.

Addition to the bath of the immune serum induced, after a delay of 15–20 min, a clear and progressive inhibition of the hemidiaphragm responses to the electrical stimulation of the phrenic nerve (Figure 1). However, the muscle responses elicited by direct electrical stimulation were not affected (Figure 1c). Immune sera obtained from different animals had different potencies in reducing the strength of the muscle contractions. The majority of the sera tested were active at concentrations of about 1%. Physostigmine induced a temporary partial reversal of the effect of the serum (Figure 1d) which was followed in some cases, by an enhancement of the block (Figure 1e). In the same preparations a further dose of physostigmine at higher concentration was also ineffective. This result could be explained by a presynaptic effect of physostigmine. Anticholinesterase drugs can induce a hyperpolarization of nerve endings (Bowman & Webb, 1972) which could impair the acetylcholine release. It is also possible that an excessive concentration of the preserved acetylcholine at the motor end plate may provoke a desensitization of the cholinoceptors (Katz & Thesleff, 1957). This phenomenon might be further enhanced by the action of antibodies on the receptors.

The effect of the sera was irreversible when its contact time with the hemidiaphragm was longer than 15 min (Figure 1d).

Inactivation of the complement of the immune sera

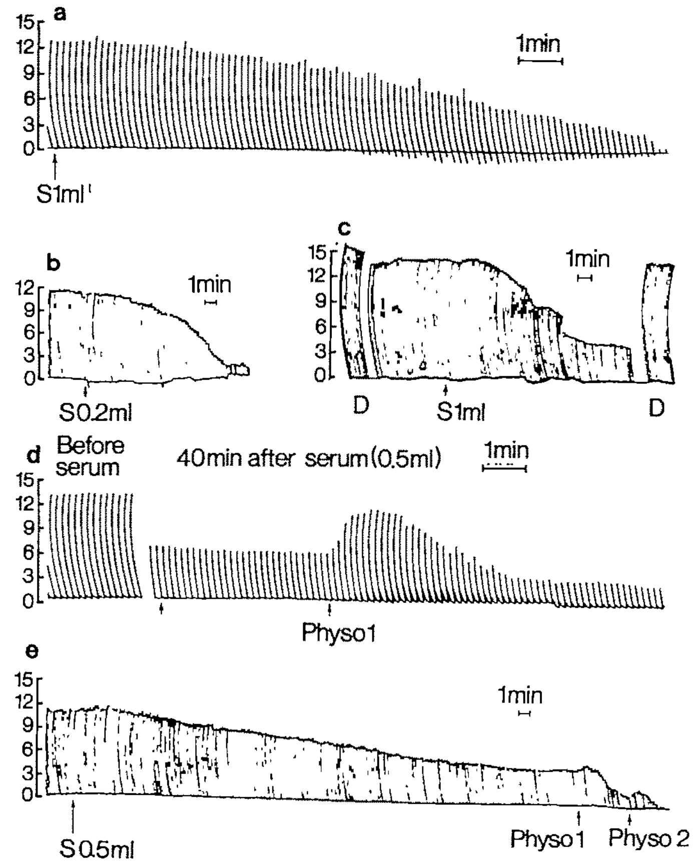

Figure 1 Effect and potency of different immune sera (S) on the rat hemidiaphragm preparation. The strength of the contractions is calibrated in grams. (a) Effect of immune serum No. 1 heated for 30 min at 56°C. (b) Effect of serum No. 2 showing a greater blocking effect. (c) Effect of immune serum No. 3. Twitches were elicited by direct (D) and indirect stimulation. The serum reduced the indirectly evoked responses without affecting significantly the responses induced by direct stimulation of the muscle. (d) Effect of physostigmine (Physo) on partially impaired contractions of rat hemidiaphragm by immune serum No. 4. Physostigmine (2.6 μM) was added to the organ bath 45 min after serum and 5 min after washing (arrow). (e) This recording demonstrates the enhancement of the blockade by physostigmine. Physo 1 = Physostigmine 2.6 μM, Physo 2 = physostigmine 13 μM.

by heating it at 56°C for 30 min did not influence their blocking activity (Figure 1a).

In order to understand whether the inhibitory activity of the immune serum was really due to its content of antibodies against the cholinoceptors, a mixture of 1 ml of active serum with 0.5 mg of receptor protein was prepared and tested 15 min later on the nerve-muscle preparation: no activity remained in such neutralized serum.

On frog rectus abdominis the immune serum displayed a curare-like activity, antagonizing, at concentrations ranging from 1% to 10%, the contraction produced by acetylcholine (Figure 2). Control sera did not show any effect on acetylcholine-induced contractions. Normal and immune sera had the same cholinesterase activity (Clementi, Conti-Tronconi, Berti & Omini, unpublished observations).

Effects of immune serum on ganglionic transmission

Since the sera were obtained by immunizing the rabbits against a nicotinic receptor, experiments were performed to investigate the specificity of the antibodies produced. It is known that nicotinic receptors are subdivided into at least two groups, since there are drugs that selectively interfere with transmission at ganglia, and other drugs which selectively inhibit neuromuscular transmission.

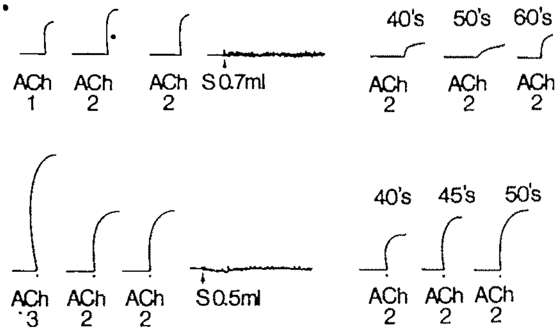

Figure 2 Serum No. 2 (S) upper panel and No. 1 (S) lower panel, show a clear antagonism, with different potencies, of the acetylcholine-induced contractions of the frog rectus abdominis preparation. The times quoted indicate the time after the addition of the serum to the preparations. Preliminary experiments showed that after 40 min of contact, the serum-induced inhibition was maximal. (The bath was washed after each response to acetylcholine). ACh1 = acetylcholine 3.4 μM, ACh2 = acetylcholine 6.8 μM, ACh3 = acetylcholine concentration 13.6 μM.

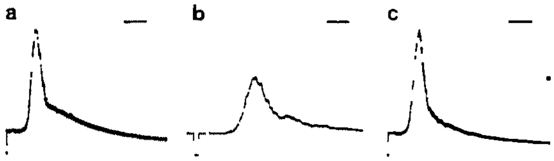

Figure 3 Recordings of compound action potential of rat superior cervical ganglion *in vitro*. The action potentials were recorded from the internal carotid nerve. (a) Control preparation. (b) Same preparation bathed 15 min with Ringer containing 5% of serum No. 2. (c) Same preparation 20 min after washing in normal Ringer. Calibration pulses are 1 mV and time marks indicate in (a) and (c) 10 ms and in (b) 20 ms.

The immune serum was tested on the isolated superior cervical ganglion of the rat. The immune sera at a concentration of 1% reduced the amplitude of the compound action potentials evoked in the superior cervical ganglion by electrical stimulation of the preganglionic trunk by 50% in about 15 min (Figure 3). Increasing the dose or the exposure time of the preparation to the serum did not lead to an increased inhibitory effect. The effect of the serum was completely removed by prolonged washing of the preparation, in contrast to the irreversible effect on the neuromuscular junction.

Effects on muscarinic receptors

When the immune sera were tested on guinea-pig ileum preparations, no inhibitory activity against spontaneous motility or acetylcholine-induced contraction was detected. However several sera, both normal and immune, induced a sustained contraction of the isolated ileum which was not antagonized by atropine. Inactivation of the complement did not alter the serum activity.

Discussion

The pharmacological analysis of sera obtained from rabbits that developed a muscular weakness after various injections of *Torpedo* acetylcholine receptor-rich fraction indicates the presence in the sera of a factor which interferes with neuromuscular transmission. In this respect the activity of the immune serum shows some similarities with curare-like drugs. This assumption is supported by the fact that on rat hemidiaphragm preparations the immune sera impaired neuromuscular transmission without affecting the muscular contractions evoked by direct stimulation. Furthermore the blockade induced by the sera could be temporarily antagonized by anticholinesterase drugs.

In addition, the antagonism exerted by immune sera against the depolarizing effect of acetylcholine on frog rectus abdominis further emphasizes the antinicotinic receptor activity of the immune serum. The effect of the immune sera seems to be specific against nicotinic receptors, since the muscarinic receptors on the ileal smooth muscle are not affected.

The reduction of the compound action potentials evoked in the superior cervical ganglion of the rat by the immune serum is only partial and is reversible, providing further evidence of steric differences between nicotinic receptors in ganglia and at the neuromuscular junction.

The curare-like activity is present only in sera containing antibodies against the cholinoceptors. This activity does not depend on the presence of complement and is unlikely to be due to non-specific effects on the *in vitro* preparations since only neuromuscular transmission is affected. Furthermore the serum neutralized with the receptor protein is devoid of any activity.

It is interesting to observe that the antibodies are active not only against the electroplax receptors (Patrick, Lindstrom, Culp & McMillan, 1973), but also against the nicotinic receptors of animals of different species. This is further substantiated by the recent reports on the possibility of inducing immune flaccid paralysis in rats and guinea-pigs (Heilbronn, personal communication) and monkeys (Tarrab-Hazdai, Aharonov, Silmann & Fuchs, 1975).

At the present time we do not know the mechanism by which the antibodies inactivate the receptor-protein. Inhibition of receptor activity may result from direct interaction of the antibody-combining sites with the active sites of the receptor molecule or from restriction in the diffusion of the agonist into the active sites by steric hindrance, or from conformational changes of the receptor molecule produced by binding of the antibody. Such mechanisms have been proposed, and partially proved, by studies on the effects of antibodies on enzyme activity (Gurari, Silmani & Fuchs, 1974). Furthermore we do not know if the antibodies as well as altering the ability of receptors to bind acetylcholine molecules also inhibit the receptor control of ionic permeability.

In conclusion the data reported here clearly support the hypothesis that the paralysis that Patrick & Lindstrom (1973) described in rabbits and that we have further examined (Clementi *et al.*, 1975) is brought about by circulating antibodies that inactivate the nicotinic receptors. Our observations could be correlated with the claims of a curarizing substance present in myasthenic sera (Berg, 1953; Nastuck *et al.*, 1959; Walker, 1973; Laurent, 1973) and with a recent finding in these patients of a γ-globulin that inhibits the binding of a specific toxin to cholinoceptors (Alman *et al.*, 1974).

Furthermore from our experiments it appears that the nicotinic receptor proteins present early in phylogenesis still maintain a close antigenic relationship with the nicotinic receptors of mammals. This is not a common phenomenon among some of the proteins at the cholinergic junction. For example, it has been observed that antibodies against choline-acetyltransferase of mammals do not cross react with the enzyme of non-mammalian species (Singh & McGeer, 1974) and antibodies produced against the *Torpedo* synaptic vesicles do not show a cross reaction against mammalian cholinergic synaptic vesicles (Ulmar & Whittaker, 1974).

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THE RELATIONSHIP BETWEEN CARDIOTOXICITY AND PLASMA DIGOXIN CONCENTRATION IN CONSCIOUS DOGS

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1 The tendency of a given oral dose of digoxin to induce cardiac dysrhythmia was determined indirectly at various times after its administration to eight conscious dogs by measurement of the intravenous dose of acetylstrophanthidin necessary to induce toxic changes in the ECG. Acetylstrophanthidin was used because its rapid elimination from the body permitted estimates to be made 45, 180 and 360 min after digoxin administration.

2 Each dog underwent four studies in which doses of 0.05, 0.1, 0.2 and 0.4 mg/kg digoxin were used in a randomized sequence allowing at least ten days between each dose.

3 Digoxin reduced the amount of acetylstrophanthidin required to cause toxic changes in the ECG; this increase in cardiac sensitivity was dose-dependent.

4 There was no correlation between plasma levels of digoxin and the tendency to dysrhythmia, since peak plasma concentrations of digoxin were reached at about 60 min after dosing whereas maximal sensitivity to acetylstrophanthidin was found 3 to 6 h after administration of digoxin.

5 These results suggest that there is little or no increased risk of cardiotoxicity during periods of transient increase in plasma levels of digoxin.

Introduction

Some oral digoxin preparations of high bioavailability produce relatively high peak plasma concentrations and it has been suggested that this could be associated with an increased incidence of toxicity in man (Harter, Skelly & Steers, 1974; Reissell, Manninen, Ojala & Karjalainen, 1974). In fact nausea can occur within 2 h of an oral dose of digoxin, i.e. at about the time of peak plasma concentration (Redfors, 1972; Falch, Teien, Bjerkelund, 1973). However, maximal positive inotropic and chronotropic effects occur several hours after oral or intravenous digoxin administration to man, presumably after tissue-plasma equilibrium has been established (Ganz, Fujimori, Penna, Greiner & Gold, 1957; Shapiro, Narahara & Taubert, 1970).

The aim of this study was to determine whether susceptibility to cardiac dysrhythmias or conduction disturbances was maximal at the time of peak plasma levels of digoxin. Toxic effects of digoxin on the heart were determined indirectly at various times after oral administration to conscious dogs by assessing the intravenous dose of acetylstrophanthidin needed to induce characteristic electrocardiographic changes.

Methods

Studies were performed on eight unanaesthetized mongrel dogs, of either sex, initially weighing 10.4–17.4 kg, and trained to lie quietly throughout the experiment. The cardiac tolerance to acetylstrophanthidin was determined in each animal by the infusion of a 100 μg/ml solution in 0.9% w/v NaCl solution (saline) and 6% ethanol via a 'Bardic I-Catheter' (C.R. Bard International Ltd) inserted into either a cephalic or saphenous vein. The rate of infusion was maintained at 95 μg/min by means of a continuous slow injector (C.F. Palmer Ltd). The ECG (lead II) was monitored continuously and the amount of acetylstrophanthidin required to induce cardiotoxicity determined.

It was accepted that cardiotoxicity had developed when one or more of these ECG changes occurred: (a) multifocal ectopics; (b) ventricular ectopic beats occurring with an equal or greater frequency than normal complexes; (c) supraventricular or ventricular tachycardia, or (d) complete heart block. When cardiotoxicity was established the infusion was stopped and the time noted for the restoration of sinus rhythm. Tolerance to acetylstrophanthidin was determined on three occasions in each study, namely at 45 (ASI), 180 (ASII) and 360 min (ASIII) after either the beginning of a control experiment or the administration of digoxin. Hartmann's Solution (Ringer-lactate) was infused at 1 drop/s for 15 min after each acetylstrophanthidin test to replace fluid losses from vomiting.

Each dog underwent four studies with oral doses of 0.05, 0.1, 0.2 and 0.4 mg/kg digoxin using a randomized sequence of administration; at least 10 days was allowed between each experiment. Digoxin stock solution containing 5 mg/ml in 70% alcohol was diluted just before administration and the diluted solution contained 1.4 to 14% ethanol. The digoxin was administered to each dog by a catheter inserted into the oesophagus and was washed in with 20 ml of water. Each dog was weighed at the beginning of each experiment. At least two control experiments with acetylstrophanthidin alone were carried out, including one at the beginning of the study, and one before the third or fourth dose of digoxin. A second 'Bardic 1-Catheter' was inserted into the contralateral cephalic or saphenous vein for sampling of blood at 0, 15, 30, 45, 60, 90, 120 and 360 min after the administration of digoxin. The plasma levels of digoxin were measured by a radioimmunoassay technique using the Lanoxitest-gamma kit (Wellcome Reagents Ltd) with an iodinated tyrosine derivative of digoxin as the tracer, but with the modification that standards were prepared with pooled dog serum. Serum potassium, calcium and urea were determined 6 h after the administration of digoxin. The results were subjected to analysis of variance, recovery times being transformed into logarithms prior to analysis.

Results

Changes in the ECG observed after infusion of acetylstrophanthidin and accepted as evidence of cardiotoxicity varied between occasions for individual dogs. This variation was seen on different days, or occasionally on the same day with different ECG changes for ASI, II or III. One dog died at ASII after receiving the 0.2 mg/kg dose of digoxin. In this dog the infusion was stopped when the desired ECG changes occurred, but the heart went into irreversible ventricular fibrillation.

Table 1 Effects in unanaesthetized dogs of oral doses of digoxin on the intravenous dose of acetylstrophanthidin (μg/kg) required to induce cardiotoxicity

Digoxin dose (mg/kg)	*Acetylstrophanthidin dose (μg/kg)* *I*	*II*	*III*
Control	135 ± 11.1	116 ± 9.2	130 ± 9.3
0.05	133 ± 15.7	97 ± 10.4	117 ± 16.9
0.1	113 ± 13.4	89 ± 11.6	107 ± 18.9
0.2	81 ± 15.5*	55 ± 11.3**	57 ± 15.8**
0.4	80 ± 16.0*	42 ± 6.1**	40 ± 5.3**

Columns I, II and III represent tolerance testing at 45, 180 and 360 min after digoxin dosing.
Mean values ± s.e. for groups of eight dogs (0.05 and 0.1 mg/kg) and seven dogs (0.2 and 0.4 mg/kg). Asterisks denote significant differences between control and treatment values at the 5% (*) and 1% (**) levels respectively.

Table 2 Effects in unanaesthetized dogs of oral doses of digoxin on the times to recovery from acetylstrophanthidin-induced toxicity

Digoxin dose (mg/kg)	*Recovery from acetylstrophanthidin (min)* *I*	*II*	*III*
Control	10.2 ± 1.3	9.9 ± 1.1	8.6 ± 1.0
0.05	8.6 ± 1.6	18.1 ± 7.2	10.3 ± 1.9
0.1	15.4 ± 4.1	15.4 ± 4.3	16.9 ± 5.9
0.2	13.5 ± 3.2	16.9 ± 4.3*	17.6 ± 5.0**
0.4	15.1 ± 3.7	19.9 ± 3.7**	25.1 ± 6.4**

Columns I, II and III represent tolerance testing at 45, 180 and 360 min after digoxin dosing.
Mean values ± s.e. for groups of eight dogs (0.05 and 0.1 mg/kg), six dogs (0.2 mg/kg) and seven dogs (0.4 mg/kg). Asterisks denote differences between control and treatment values significant at the 5%(*) and 1% (**) levels.

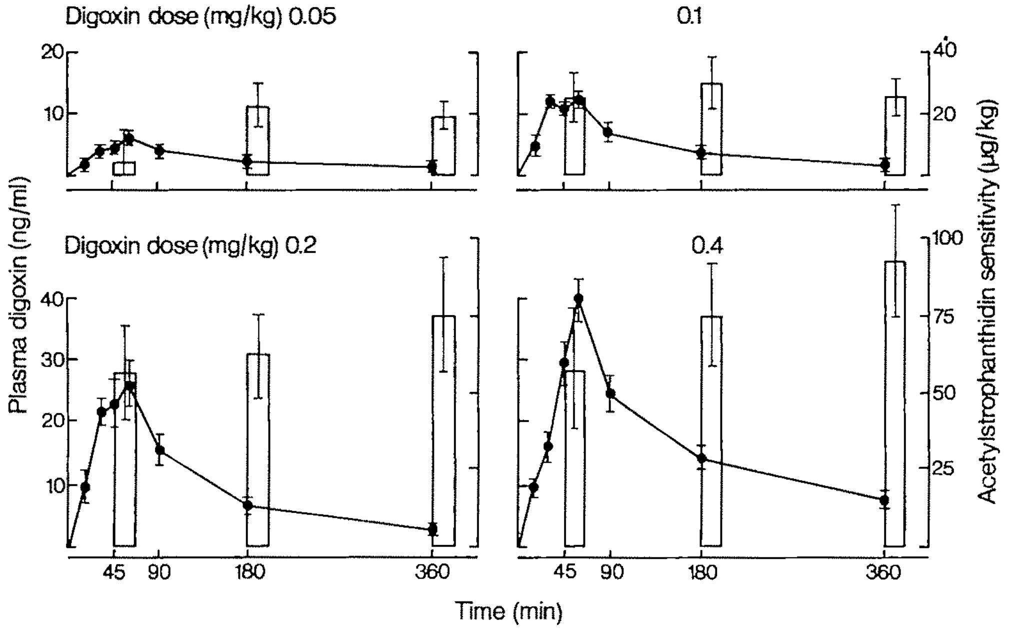

Figure 1 Comparisons of mean plasma digoxin concentration and mean acetylstrophanthidin sensitivity (see text) for four oral doses of digoxin administered to unanaesthetized dogs. The plasma digoxin levels are shown by the curves whilst acetylstrophanthidin sensitivity is represented by the columns. Vertical lines represent s.e. mean. The mean plasma digoxin concentrations are taken from Table 3.

The amount of acetylstrophanthidin per kg of body weight that was required to produce cardiotoxicity is shown in Table 1. The control doses of acetylstrophanthidin were calculated for each administration (ASI, II or III) for each dog and these doses varied more between dogs than between each administration. However, a small but significant reduction in acetylstrophanthidin tolerance was noted for ASII. Retching and vomiting usually occurred before cardiotoxicity, but the associated electrocardiographic changes were normally of short duration.

Treatment with digoxin reduced the amount of acetylstrophanthidin required to cause cardiotoxicity and this increased susceptibility to toxicity was dose-dependent (Table 1). At the lower dose levels of 0.05 and 0.1 mg/kg digoxin, increased susceptibility was most obvious at ASII, though not significant. At the two higher dose levels of 0.2 and 0.4 mg/kg of digoxin, reduced acetylstrophanthidin tolerance was maximal at ASII, persisted to ASIII, and differed significantly from the control experiments.

The time required for the ECG to return to normal after treatment with acetylstrophanthidin differed between dogs in the control experiments, but there was no significant difference between each administration of acetylstrophanthidin (ASI, ASII, ASIII) in individual dogs (Table 2). In one dog which received 0.2 mg/kg digoxin, acetylstrophanthidin-induced toxicity was abnormally prolonged; ectopic beats persisted for 130 min after ASI and 165 min after ASII, and ventricular tachycardia persisted for 48 h after ASIII. These data were excluded from analysis.

Table 3 Plasma digoxin concentration (ng/ml) after oral administration of digoxin

Digoxin dose	*Time (min) after administration*							
(mg/kg)	*0*	*15*	*30*	*45*	*60*	*90*	*180*	*360*
0.05	0	1.8±0.7	3.8±0.9	4.4±0.7	5.8±1.3	3.8±0.8	1.9±0.8	0.7±0.2
0.1	0	4.7±1.8	11.6±1.2	10.4±1.0	11.9±1.5	6.6±1.7	3.3±0.7	1.5±0.4
0.2	0	9.6±3.5	22.0±3.5	23.1±5.4	26.2±5.6	15.5±3.5	6.7±1.9	2.7±0.8
0.4	0	9.9±2.2	16.3±3.5	29.9±4.6	40.5±4.7	25.0±4.2	14.4±2.5	7.3±1.6

Mean values ± s.e. for group of eight dogs are shown for various times after dosing.

Recovery from acetylstrophanthidin-induced toxicity tended to be prolonged with increasing dosage of digoxin. This prolongation was least obvious at ASI and the recovery times were only significantly greater than the controls at ASII and ASIII after the two higher doses of 0.2 and 0.4 mg/kg digoxin.

The peak plasma concentration of digoxin was reached at about 60 min after each dose and then declined steadily (Table 3). In these dogs, vomiting did not occur until the first infusion of acetylstrophanthidin which was started 45 min after administration of digoxin. The occurrence of vomiting after the first infusion of acetylstrophanthidin depended on the dose of digoxin administered, i.e. for 0.05, 0.1, 0.2 and 0.4 mg/kg digoxin the mean times for the onset of vomiting from starting the infusion of acetylstrophanthidin were 15, 11, 7 and 6 min respectively.

Acetylstrophanthidin sensitivity was calculated as the difference between the amounts of acetylstrophanthidin required to induce cardiotoxicity in the digoxin and the control studies. The calculations were carried out from the results for comparable acetylstrophanthidin infusions (ASI, II or III) for individual dogs. Comparison of the plasma levels of digoxin and acetylstrophanthidin sensitivity showed that there was no correlation between these two variables (Figure 1). This was most evident after the 0.2 and 0.4 mg/kg doses of digoxin.

There was no significant change in serum calcium, potassium or urea during any experiment. The individual maximal fall in serum potassium from initial to 6 h blood sample was 0.2 mEq/litre.

Discussion

The absorption of digoxin occurs rapidly from the upper part of the gastrointestinal tract; peak plasma concentrations usually appearing after about 1 h in man (White, Chamberlain, Howard & Smith, 1971). Interest in peak concentrations arose from the observation that tablets of higher bioavailability produced an earlier and more prominent peak concentration (Johnson, Fowle, Lader, Fox & Munro-Faure, 1973). Concern was expressed that such high concentrations, although transient, might produce cardiotoxicity (Falch *et al.*, 1973; Harter *et al.*, 1974; Reissel *et al.*, 1974).

One method devised to detect the early stages of developing cardiotoxicity used acetylstrophanthidin (Lown & Levine, 1954). The effects of this synthetic cardiac glycoside are additive to those of digoxin at myocardial receptors, so that the quantity which must be infused before ECG signs of cardiotoxicity occur may be used as an index of the tendency to digoxin toxicity. The value of acetylstrophanthidin is that its additive cardiac effects are rapid in onset and highly transient, its plasma elimination half-life being 1.2 h in dogs and 2.3 h in man (Selden, Klein & Smith, 1973). This is much shorter than that of any other digitalis analogue and the decay of its pharmacological effect is even more rapid (Klein, Nejad, Lown, Hagemeijer & Barr, 1971). This relatively rapid rate of elimination allows repeated estimates of acetylstrophanthidin tolerance. Control experiments showed no important change in acetylstrophanthidin tolerance between the first and third infusions, nor was there any increase in the time for recovery of sinus rhythm.

In this work some aspects of the cardiotoxicity associated with the infusion of acetylstrophanthidin differed from those reported by others. Ventricular tachycardia was not the only dysrhythmia, as has been suggested (Barr, Smith, Klein, Hagemeijer & Lown, 1972; Kumar, Hood, Gilmour & Abelmann, 1972). Indeed Cope, Hopkins & Taylor (1973) also showed that the nature of the tachycardia or conduction block may vary. Higher doses of acetylstrophanthidin were required to produce cardiotoxicity in our studies than in those noted above, possibly because our dogs were unmedicated whereas anaesthetized or sedated dogs were used by others. Furthermore the infusion rates of acetylstrophanthidin varied in these previous studies and this must be of great importance in view of its rapid elimination.

In dogs, Barr *et al.* (1972) showed that ventricular tachycardia frequently developed spontaneously after intravenous administration of 0.1 mg/kg digoxin and persisted for between 0.5 to 3 h; also that acetylstrophanthidin tolerance was somewhat greater at 6 than at 2 hours. These results suggest that digoxin accumulates at myocardial receptor sites over periods of 1 to 2 h after injection and that deterioration in its activity can be detected by 6 hours. In the present study, ventricular tachycardia did not develop spontaneously after digoxin given orally, but it was readily induced by acetylstrophanthidin, particularly after the 0.2 and 0.4 mg/kg doses of digoxin, i.e. the susceptibility to acetylstrophanthidin was dependent upon the magnitude of the preceding dose of digoxin. However, at all doses of digoxin, acetylstrophanthidin sensitivity was lowest 45 min after oral administration of digoxin, although at this time plasma concentrations were at or near their maximum. Cardiac toxicity was always more easily induced at either 3 or 6 h after dosing with digoxin and there was a suggestion that maximal sensitivity might occur later with higher doses (Figure 1). It is clear that the greater tendency to cardiotoxicity occurs not at maximum plasma concentration of digoxin but some hours later. It is possible that this delay may be somewhat longer after oral than after intravenous administration. Continuing intestinal absorption might lead to the cardiac effects being more prolonged after oral administration, and this may account for acetylstrophanthidin sensitivity being greatest 6 h after the two larger doses of 0.2 and 0.4 mg/kg digoxin. The time for recovery to sinus rhythm was a less sensitive index of cardiotoxicity than the dose of acetylstrophanthidin required to induce toxicity. However, the same general trend was noted, as recovery from acetylstrophanthidin was more delayed when given 3 to 6 h after the higher doses of digoxin.

Acetylstrophanthidin induced vomiting but electrolytes were appropriately replaced by Hartmann's solution, since serum potassium, calcium and urea were not significantly altered during the studies. Although a change in serum electrolytes can alter the sensitivity of the heart to cardiac glycosides, this was unlikely in these experiments.

Dogs absorb digoxin well, and respond to and eliminate it at rates comparable to those of man (Barr *et al.*, 1972). They may also have similar processes of tissue-plasma kinetic equilibrium. With this assumption, the reported studies suggest that cardiotoxicity will not be temporally related to peak plasma concentrations in man. The rate of myocardial uptake of digoxin in dogs is linearly related to its plasma concentration (Roberge, Marcus, Kapadia & Lown, 1970). It may be that some part of the greater effectiveness of high bioavailability preparations is related to the exaggerated peak plasma concentrations. However, only a fraction of digoxin taken up by the myocardium is pharmacologically effective (Lüllman & Van Zwieten, 1969) and the relation of the plasma concentration of digoxin and this active myocardial fraction is unknown. It is also unknown whether the qualitative difference in plasma concentration profile resulting from different oral digoxin preparations is associated with variation in cardiac response. However, it is clear that cardiac response to digoxin is not transient but is delayed and prolonged.

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EMETINE MYOPATHY IN THE RAT

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1 (–)-Emetine (0.25–2.0 mg/kg i.p.) was administered to rats for up to 220 days.

2 At doses of 1.0 mg/kg or less, the animals continued to gain weight but more slowly than the untreated control animals. The physiological changes in the muscles from these animals were minimal; there was a small reduction in both the resting membrane potential and in the maximum rate of rise of the action potential. There was no atrophy or loss of muscle fibres although in the occasional muscle, hyaline fibres, necrotic fibres and split fibres were observed. There was a focal loss of myofibrillar adenosine triphosphatase (ATPase) and nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) in Type II and Type III fibres, but no such loss in Type I fibres.

3 The animals receiving 2.0 mg/kg of (–)-emetine gained weight slowly for up to 20 days but then rapidly lost weight and by 30 days they were weak and emaciated. The muscles from these animals were severely atrophied and the total muscle wet weight was reduced by almost 20%.

4 The strength of the muscles from these animals was measured *in vitro* using direct stimulation. They were weaker than normal both in absolute terms and when expressed in terms of tension developed/unit wet weight.

5 There was no evidence of either functional or structural denervation but surgically denervated muscles from animals in this group were indistinguishable from denervated muscles from normal rats.

6 Severe structural damage was obvious in the fibres of both extensor digitorum longus and soleus. Necrotic, hyaline and splitting fibres were common and the focal loss of myofibrillar ATPase and NADH-TR activity was extensive and occurred in Type I fibres as well as in Type II and Type III fibres.

7 It is concluded that the muscular weakness induced by (–)-emetine is due to a direct effect on the muscle fibres and that this occurs at a subcellular level. There is no evidence that functional or structural denervation plays any role in the aetiology of emetine myopathy in the rat.

Introduction

The drug (–)-emetine, isolated from the root of *Cephaelis ipecacuanha*, has been used for more than 50 years in the clinical management of amoebiasis (Woolfe, 1963, 1965). The drug has also been used for the control of amoebiasis in experimental animals infested with *Entamoeba histolytica* (Williams, 1959).

Muscle weakness is a recognized side effect of the prolonged administration of the drug (Klatskin & Freidman, 1948; Fewings, Burns & Kakulas, 1973) and the symptoms have been graphically described by Young & Tudhope (1926). The precise cause of the weakness is not known, and it has been variously reported that emetine is capable of blocking transmission at ganglia and neuromuscular junctions (Ng, 1966; Salako, 1970) and that it is capable of directly damaging skeletal muscle fibres 'without recognizable morphological or physiological alteration in the neural components of the motor unit' (Duane & Engel, 1970). However, the role of the peripheral nervous system in the pathogenesis of (–)-emetine-induced muscle weakness is difficult to study because, under certain circumstances, the drug is capable of inhibiting protein synthesis (Grollman, 1966, 1968; Johnson, Donahue & Jondorf, 1971). Inhibitors of protein synthesis may delay the appearance of denervation-induced changes in mammalian skeletal muscle (Grampp, Harris & Thesleff, 1971; Llados & Zapata, 1974). Thus the absence of fibrillation potentials from the muscles of (–)-emetine-poisoned animals (Duane & Engel, 1970) does not necessarily imply that the peripheral nerve is intact and functioning normally. Moreover, in some instances, a morphologically normal motor nerve supply may be associated with a muscle exhibiting many of the usually accepted 'denervation-like' changes (Thesleff, 1960; Josefsson & Thesleff, 1961; Duchen & Stefani, 1971; Harris & Ward, 1974).

In this paper we describe our observations on the effect of (–)-emetine intoxication on the histology and physiology of mammalian skeletal muscle, and our attempts to clarify the role of the nerve in the development of the syndrome.

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Methods

Animals

Male Wistar rats were used which weighed 145–210 g at the start of the experiments. They were housed 6–8 per cage. They were allowed free movement and continual access to food and water. Each animal was weighed weekly and was subjected to regular clinical examination.

The animals were divided into 6 groups, each group comprising 12–24 animals. One group served as a normal control. The remaining groups of animals were given (–)-emetine by intraperitoneal injection for 5 successive days in each week, at a dose level of 0.25, 0.50, 0.75, 1.0 or 2.0 mg/kg respectively.

Animals receiving (–)-emetine, 0.25–1.0 mg/kg, were killed between 120 and 240 days after the start of the experiment; animals receiving 2.0 mg/kg were killed 21–30 days after the start of the experiment. The heart and pleural cavity were examined for obvious signs of cardiac or pulmonary distress. The muscles, usually extensor digitorum longus (EDL) and soleus (SOL), were then removed for physiological, histological or histochemical examination.

Physiology

Experiments *in vivo* were made on tibialis anterior muscles. The animals were anaesthetized with urethane (1.5–2.0 g/kg i.p.). The sciatic nerve was exposed in the thigh and all muscles other than the extensor muscles were denervated. The tibialis anterior muscle was exposed by removing the covering skin and connective tissue. The animals were placed in a bath containing Liley's (1956) bathing fluid (composition (mM): Na^+ 150; K^+ 5.0, Ca^{2+} 2.0, Mg^{2+} 1.0, Cl^- 148, $H_2PO_4^-$ 1.0, HCO_3^- 12.0, glucose 11.0), maintained at room temperature and equilibrated with 95% O_2 and 5% CO_2. The distal stump of the sciatic nerve was mounted on a pair of silver wire electrodes and stimulated with supramaximal rectangular stimuli of 0.05–0.10 ms duration. Action potentials, generated by the application of a single stimulus to the nerve, were recorded from single muscle fibres using an intracellular recording technique (q.v.). Action potentials generated by the application of repetitive stimuli (50 Hz for 200–250 ms) to the nerve were recorded from the muscle with a concentric needle electrode (Medelec, 0.5 mm diameter).

Experiments *in vitro* were made on isolated EDL and SOL muscles. The muscles were mounted in a constant flow bath which was continuously perfused with gassed Liley's fluid maintained at 30–32°C.

Resting membrane potentials were recorded with intracellular microelectrodes. The electrodes were pulled from glass tubing (Corning Ltd, Type 7740) and were filled with 3M KCl. They had tip potentials less than 5 mV and d.c. resistances of 5–15 MΩ. The membrane potential was recorded using a unity gain input stage (input impedance, $10^{12}\,\Omega$) and an oscilloscope. Action potentials were generated and recorded by a double microelectrode technique. The current passing electrode, filled with 3M KCl, was inserted into the same fibre as the recording electrode at a separation of 50–100 µm. The muscle fibre membrane was locally hyperpolarized to between –90 and –95 mV before passing depolarizing current (cf. Redfern & Thesleff, 1971). Capacitance neutralization was achieved using feedback between the output and the input of the buffer amplifier. The time differential of the action potential was obtained by electronic differentiation. The frequency response of the differentiator was flat to frequencies in excess of 5 KHz.

Miniature end-plate potentials were recorded from putative end-plate regions by the described intracellular techniques. The noise level of the recording apparatus was approximately 150 µV peak to peak.

The ability of muscles to develop a contracture in response to the application of acetylcholine was assessed under isometric conditions. The muscles were mounted vertically in a 10 ml bath of gassed Liley's fluid at room temperature. A resting tension of 5.0 g was routinely used. Tension was measured with a strain gauge (Statham, G3). Extrajunctional sensitivity to acetylcholine was also examined by the technique of iontophoresis (Nastuk, 1953; Axelsson & Thesleff, 1959).

The ability of the (–)-emetine-poisoned muscles to develop tension in response to direct stimulation was measured *in vitro* under isometric conditions. The EDL muscles were mounted horizontally in a moist chamber on a multi-electrode assembly at a resting tension of 5.0 grams. The electrodes were platinum wires, 2.5 mm apart, assembled as alternating anode and cathode. Stimulation was with 90 V shocks of 1.0 ms duration at a frequency of 150–200 Hz and at room temperature. The responses were recorded with a strain gauge (Statham G1-8-350) and were displayed directly on a storage oscilloscope.

In some experiments we wished to study the effects of denervation on (–)-emetine-poisoned muscles. In these cases, EDL muscles were denervated by sectioning the common peroneal nerve at the knee. The muscles were removed 4 days later.

Histology and histochemistry

Blocks of tissue comprising the middle 4 mm of EDL and SOL muscles were sandwiched between thin slices of liver and orientated so that transverse sections could be obtained. The tissue blocks were then frozen in dichloro-difluoromethane, maintained at approximately −150°C in liquid N_2. The transverse sections, 10 µm thick, were stained with haematoxylin and eosin and sections serial to these were used to demonstrate the activity of Ca^{2+}-activated myofibrillar adenosine triphosphatase (ATPase) (Hayashi & Freiman, 1966) and nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR, Pearse, 1972). Where appropriate the following histochemical methods were also applied: acid phosphatase (Burstone, 1958a, b), myophosphorylase (Godlewsky, 1963), periodic acid-Schiff (McManus, 1948), myofibrillar ATPase after acid-preincubation (Brooke & Kaiser, 1969).

The system of fibre type nomenclature adopted in this study is based on the properties of myofibrillar ATPase in rat skeletal muscle as defined by Stein & Padykula (1966). In rat EDL there is a relatively small number of fibres (approximately 2%) which have low myofibrillar ATPase and high levels of mitochondrial oxidative enzymes (Type I fibres). The remaining fibres consist of approximately equal numbers of small diameter fibres with high myofibrillar ATPase resistant to formalin fixation (Type III), and larger diameter fibres whose myofibrillar ATPase is more sensitive to the effects of formalin (Type II). Thus, the use of the technique for demonstrating myofibrillar ATPase as described by Hayashi & Freiman (1966) makes it possible to differentiate these fibre types on the basis of a single enzyme reaction.

Table 1 Correlation of enzyme characteristics and muscle fibre type in rat extensor digitorum longus muscles

Fibre type	*Myofibrillar ATPase activity (after formalin fixation)*	*Mitochondrial oxidative enzyme activity*
I	+	++
II	++	+
III	+++	++

The mitochondrial oxidative enzyme activity of the Type III fibres is approximately equal to that of the Type I fibres, whereas the Type II fibres have a much lower activity of oxidative enzymes. The correlation of the enzyme characteristics of the three fibre types is summarized in Table 1.

Drugs and chemicals

(−)-Emetine was used as the hydrochloride, and all doses refer to this salt. It was obtained in pure form from BDH Ltd and made up into solution in pyrogen-free distilled water. It was supplied by the Pharmacy Department, Newcastle General Hospital in the form of sterilized sealed 1.2 ml ampoules of 40 µg/ml.

Tetrodotoxin (Sankyo Chemical Co., Tokyo) was supplied by Koch-Light Laboratories Ltd. All other drugs and chemicals were obtained from the usual commercial sources.

Statistical treatment of results

Where necessary, the significance of the difference between two means was assessed with Students' *t* test; a level of significance of $P < 0.05$ was used.

Results

Clinical observations

All animals receiving (−)-emetine at dose levels of 0.25–1.0 mg/kg gained weight, although not quite as rapidly as normal. The animals receiving 2.0 mg/kg ceased to grow as soon as drug administration began, and by 14–20 days began to lose weight. Details of mean body weight of all groups of animals are given in Table 2. In Figure 1 the mean weight of selected groups of animals are plotted, with all weights expressed relative to the initial body weight (which has been termed 100%). It can be seen that animals receiving a low dose of (−)-emetine gain weight normally for about 6 weeks, and only then begin to fall

Table 2 The mean body weight (± s.e.) of the various groups of animals obtained at different times after the start of the experiments; animals were assigned to either a control group or to a group receiving emetine, 5 days in every 7, at the stated dose

Time	*Control*	*0.25 mg/kg*	*0.50 mg/kg*	*0.75 mg/kg*	*1.0 mg/kg*	*2.0 mg/kg*
0	155 ± 9.5	157 ± 2.3	160 ± 2.8	182 ± 4.1	181 ± 1.9	201 ± 4.7
2	213 ± 13.0	224 ± 13.9	227 ± 5.1	235 ± 4.2	233 ± 4.1	202 ± 4.3
4	276 ± 14.5	273 ± 4.9	277 ± 5.2	291 ± 7.2	277 ± 5.5	175 ± 9.4
6	308 ± 15.0	314 ± 6.0	309 ± 6.0	306 ± 7.3	284 ± 5.3	—
12	370 ± 16.3	370 ± 6.3	359 ± 7.0	340 ± 9.9	298 ± 5.2	—
18	434 ± 16.7	414 ± 8.8	395 ± 7.0	358 ± 9.6	298 ± 8.8	—
28	474 ± 19.0	444 ± 10.0	432 ± 14.0	379 ± 11.2	312 ± 6.5	—

Each group contained 12–24 animals

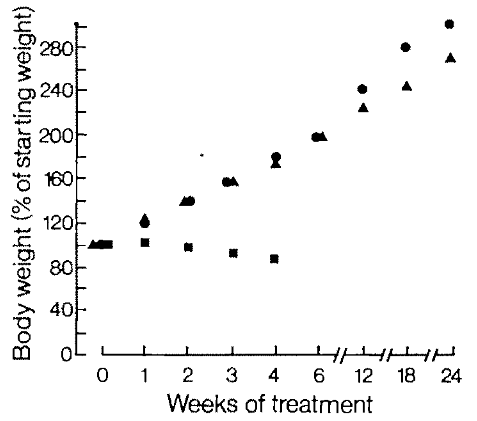


Figure 1 Growth curves of normal rats (●), rats treated with emetine 0.5 mg/kg 5 days per week (▲) and rats treated with emetine, 2.0 mg/kg 5 days per week (■). For each group of animals, the weight at time zero has been designated 100% and its weight thereafter referred to that standard.

behind normal animals. We have never observed the acute weakness following the injection of (–)-emetine that was reported by Salako (1970).

Physiology

The resting membrane potential and the characteristics of the directly elicited action potential were recorded from isolated EDL and SOL muscle fibres. The muscles were removed from control animals and animals that had received (–)-emetine at dose levels of 0.50 mg/kg and 2.0 mg/kg respectively.

(–)-Emetine administration caused a significant fall in the mean resting membrane potential of muscle fibres of both EDL and SOL muscles. The fall was larger in the muscles removed from animals that had received (–)-emetine at a dose level of 2.0 mg/kg rather than 0.5 mg/kg, even though the higher dose was given for a much shorter time period.

Action potential generation was also affected by (–)-emetine administration. Thus in EDL muscle fibres, the mean maximum rate of rise of the action potential, and the amplitude of the overshoot were reduced in muscles from animals treated at dose levels of (–)-emetine of either 0.5 mg/kg or 2.0 mg/kg, the muscles from animals receiving the higher dose being the more severely affected. In SOL muscle fibres, the maximum rate of rise of the action potential was reduced significantly only in those muscles removed from animals treated with (–)-emetine, 2.0 mg/kg. Treatment with (–)-emetine had no effect on the amplitude of the overshoot of the action potential in SOL muscle fibres. The threshold for action potential generation was increased in EDL muscle fibres after treatment with (–)-emetine at either dose level; in SOL muscle fibres, the change in threshold was significant only in those muscles removed from animals treated with the higher dose of (–)-emetine. These results are summarized in Table 3, and some representative records of action potentials are shown in Figure 2.

Miniature end-plate potentials in the (–)-emetine-treated muscles were recorded from all fibres penetrated in the end-plate region (a total of 60 fibres in EDL muscles and 40 in SOL muscles). The

Table 3 Some properties of muscle fibres in isolated extensor digitorum longus (EDL) and soleus (SOL) muscles from normal rats and rats treated with emetine, 0.5 mg/kg and 2.0 mg/kg respectively

			Action potential		
		Membrane potential (mV)	*Max. rate of rise (V/s)*	*Overshoot (mV)*	*Threshold (mV)*
Normal rats	EDL	−77.6 ± 0.4 (30)	856 ± 17 (30)	43.0 ± 0.7 (30)	51.9 ± 0.4 (30)
	SOL	−71.7 ± 0.7 (32)	660 ± 14 (32)	32.1 ± 0.9 (32)	50.6 ± 0.5 (32)
Rats treated with emetine (0.5 mg/kg)	EDL	−71.4 ± 1.2* (27)	792 ± 25* (27)	35.9 ± 1.4* (27)	49.5 ± 0.4* (27)
	SOL	−68.5 ± 0.7* (20)	629 ± 21 (20)	30.9 ± 1.3 (20)	50.9 ± 0.7 (20)
Rats treated with emetine (2.0 mg/kg)	EDL	−65.6 ± 0.8* (54)	419 ± 19* (55)	33.5 ± 0.6* (54)	41.5 ± 0.8* (42)
	SOL	−64.5 ± 0.8* (30)	430 ± 14* (30)	33.2 ± 0.9 (30)	43.1 ± 0.6* (30)

Figures quoted are means ± s.e. mean. The number of fibres sampled is given in parentheses.
*Value differs significantly ($P < 0.05$ or better) from normal.

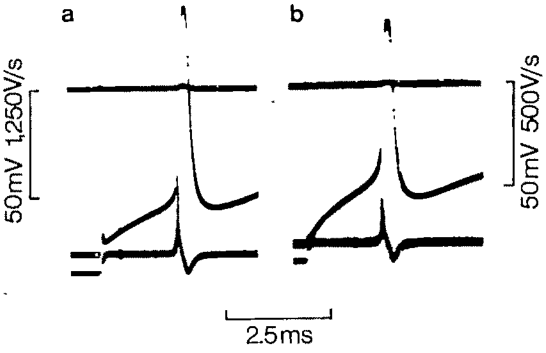


Figure 2 Muscle fibre action potentials generated and recorded by a double-microelectrode technique in isolated extensor digitorum longus muscles from (a) a normal rat and (b) an emetine (2.0 mg/kg)-treated rat. The top trace (horizontal) represents zero potential, the middle trace the first derivative of the voltage recording and the bottom trace the muscle fibre membrane potential. In each fibre, the membrane potential was artificially set to −90 mV before the passage of depolarizing current. Note the different vertical calibration bars in this figure.

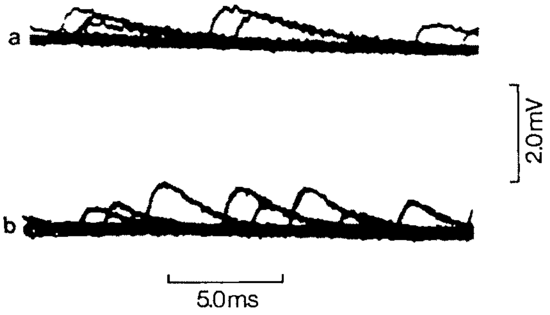


Figure 3 Miniature end-plate potentials recorded from muscle fibres in extensor digitorum longus muscles from (a) a normal rat and (b) an emetine (2.0 mg/kg)-treated rat. The recordings were made onto a storage oscilloscope, and several traces have been superimposed.

miniature end-plate potentials were generally of normal amplitude and frequency (Figure 3) although in some fibres of SOL and EDL muscles from animals treated with (−)-emetine 2.0 mg/kg, the miniature end-plate potentials exceeded 1 mV in amplitude. This is rather larger than normal (0.2–0.6 mV: these laboratories, unpublished) and probably reflects the atrophy that was noted in many of these muscles. The relationship between fibre diameter and miniature end-plate potential amplitude was demonstrated unequivocally by Katz & Thesleff (1957) to be the consequence of the relationship between the 'input resistance' of a muscle fibre and its diameter. It was of interest therefore, to note that the input resistance of EDL muscle fibres from animals treated with (−)-emetine 2.0 mg/kg was increased from a normal value of 0.426 ± 0.020 MΩ to 1.259 ± 0.10 MΩ ($n = 50, 30$, respectively). The distribution of these results is shown in Figure 4.

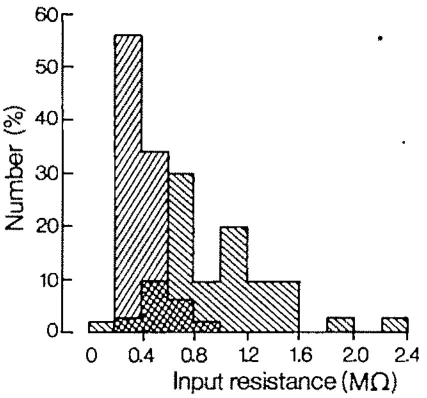


Figure 4 The distribution of 'input resistance' measurements of muscle fibres in extensor digitorum longus muscles from normal rats (cross hatch right to left) and from emetine (2.0 mg/kg)-treated rats (cross hatch left to right).

It has been suggested (Ng, 1966) that the cause of muscular weakness seen during (−)-emetine therapy is an action of (−)-emetine 'at or near the neuromuscular junction' and Salako (1970) has suggested that the drug has a 'tubocurarine-like action' on the neuromuscular junction. If an action at either a pre-synaptic or a post-synaptic site were primarily responsible for muscle weakness, the implication would be that either individual muscle fibres or entire motor-units were paralysed. Moreover this paralysis would have to be of considerable duration in order to account for the clinical features of the weakness. Such affected muscle fibres would then resemble the 'functionally denervated' muscle fibres seen, for example, in botulinum poisoning (Thesleff, 1960), inherited motor and end-plate disease (Duchen, 1970) and α-bungarotoxin poisoning (Chuang & Huang, 1975). In all of these conditions, the muscle fibres become sensitive to acetylcholine at extrajunctional regions, and generate action potentials in the presence of tetrodotoxin (Thesleff, 1960; Duchen & Stefani, 1971; Harris & Ward, 1974; Chang *et al.*, 1975). It may be of significance therefore, that we could find no 'tetrodotoxin-resistant' action potentials in the muscles of animals receiving 0.5 mg/kg (−)-emetine (20 fibres tested in each of EDL and SOL), and of a total of 87 fibres tested in muscles of rats treated with 2.0 mg/kg (−)-emetine, we found only 2 fibres (both in EDL muscles) which would generate such an action potential. The potentials were very small, and would probably not have been propagated along the muscle fibre outside the hyperpolarized region of membrane (see methods section) due to inactivation.

Moreover, extrajunctional sensitivity to acetylcholine was found to be absent when the muscles were examined by the technique of iontophoresis, and no contracture could be obtained in the muscles of (—)-emetine-treated animals even in response to acetylcholine, 0.1 mM.

These results indicated that the administration of (—)-emetine at doses large enough to cause emaciation in the animal and marked changes in the properties of the muscle fibres did not induce 'denervation-like' changes in the muscle. Further evidence that the peripheral nerve was not severely damaged was obtained by recording the electrical activity of tibialis anterior muscles *in vivo* in response to indirect stimulation in anaesthetized animals that had received (—)-emetine 2.0 mg/kg. In the first series of experiments, intracellular action potentials were recorded. All muscle fibres tested responded to indirect stimulation by generating an action potential, and the latency between stimulation and excitation of the muscle was normal. As in the experiments *in vitro*, the resting membrane potential and the maximum rate of rise of the action potentials were reduced. The amplitude of the overshoot of the action potentials was not significantly reduced. The results are summarized in Table 4.

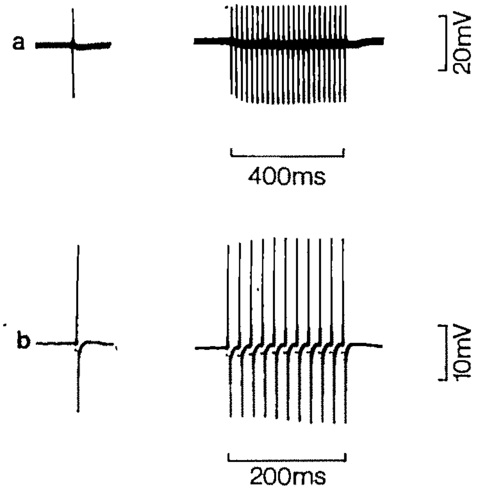


Figure 5 EMG records from the tibialis anterior muscles of two emetine (2.0 mg/kg)-treated rats (a, b) *in vivo*. A standard concentric needle electrode technique was used. The responses of the muscle to single shocks (left) and repetitive stimulation (50 Hz) are shown. The small increase in total EMG amplitude during stimulation is normal. The animals were anaesthetized with urethane.

In the second series of experiments, the electrical response of the muscle was recorded using standard EMG techniques in response to stimulation of the nerve at a frequency of 50 Hz (see methods section). The EMG revealed no 'myasthenia-like' response (see Simpson, 1966) indicating that under normal circumstances, there was little likelihood that muscular weakness would be caused by a low safety-factor of neuromuscular transmission. Some typical records are shown in Figure 5.

We next considered the possibility that either an acute or sub-acute denervating process might not give rise to physiological and pharmacological symptoms of denervation as a result of the protein-synthesis-inhibiting action of (—)-emetine. Accordingly, the

Table 4 Some properties of tibialis anterior muscle fibres *in vivo* in normal rats and in rats treated with emetine, 2.0 mg/kg.

	Muscles in normal rats	*Muscles in rats treated with emetine, 2.0 mg/kg*
Membrane potential (mV)	−78.9 ± 0.6 (24)	−71.9 ± 1.5* (22)
Action potential max. rate of rise (*V/s*)	545 ± 19 (24)	433 ± 29* (22)
Action potential overshoot (mV)	34.0 ± 2.2 (24)	28.5 ± 3.1 (22)
Action potential latency (ms)	3.10 ± 0.14 (24)	3.24 ± 0.14 (22)

Animals were anaesthetized with methane. Figures quoted are means ± s.e. mean. The number of fibres sample is given in parentheses.

* Value differs significantly ($P < 0.05$ or better) from normal.

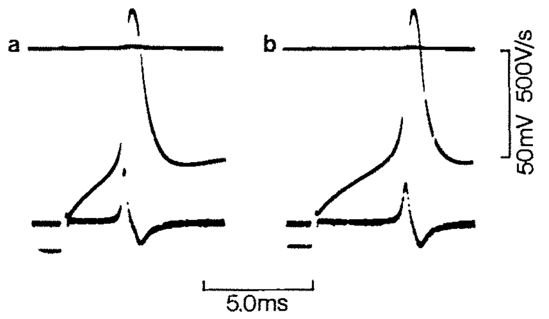


Figure 6 Typical denervated muscle fibre action potentials recorded from isolated extensor digitorum longus muscles *in vitro* in the presence of tetrodotoxin, 1 μM. (a) Record from a denervated muscle of a normal rat; (b) record from a denervated muscle of an emetine (2.0 mg/kg)-treated rat.

extensor muscles of one hind limb of six animals were denervated by sectioning the common peroneal nerve at the knee. Two animals were untreated controls, two animals had received (—)-emetine 0.5 mg/kg and two had received (—)-emetine 2.0 mg/kg. Four days after the operation the denervated muscles were removed for examination. The results (Table 5) made it clear that treatment with (—)-emetine is not able to inhibit the onset of denervation-induced changes in the muscle. Some typical tetrodotoxin-resistant action potentials are shown in Figure 6.

Since the muscles of the most severely affected animals were atrophied we wished to determine whether or not muscles were physically 'weaker' than normal when 'strength' was expressed as tension developed/unit wet weight of muscle. Tetanic contractions were elicited in isolated directly stimulated EDL muscles from normal rats and rats treated with (—)-emetine 2.0 mg/kg. The results (Table 6) show that the muscles from (—)-emetine-treated animals were not only weak, but were unable to generate the same tension as normal when expressed on the basis of their individual wet weights.

Table 6 Tetanic tension generated by isolated directly stimulated extensor digitorum longus muscles from normal rats and rats treated with emetine, 2.0 mg/kg, expressed in terms of muscle wet weight

	Muscles from normal rats	*Muscles from rats treated with emetine, 2.0 mg/kg*
Tetanic tension (g/mg wet wt.)	0.93 ± 0.03	0.60 ± 0.05*
n	7	4

Figures quoted are means ± s.e. mean. n = number of muscles studied.

*Value differs significantly ($P<0.05$ or better) from normal.

Table 5 Some properties of muscle fibres in isolated denervated extensor digitorum longus muscles from normal rats and from rats treated with emetine 0.5 mg/kg and emetine 2.0 mg/kg. The muscles were removed 220 days and 29 days respectively after treatment started. The muscles were denervated 4 days before the experiment.

	Muscles from normal rats	Muscles from rats treated with emetine	
		0.5 mg/kg	*2.0 mg/kg*
Membrane potential (mV)	−62.0 ± 0.5 (11)	−62.4 ± 0.6 (10)	−60.0 ± 1.3 (21)
Action potential			
Max. rate of rise (*V/s*)	582 ± 36 (11)	530 ± 42 (10)	376 ± 20 (21)
Overshoot (mV)	30.6 ± 2.0 (11)	33.0 ± 1.4 (10)	26.0 ± 0.3 (21)
Action potential in tetrodotoxin† occurence*	14/14	14/14	16/16
Max. rate of rise (*V/s*)	212 ± 8 (14)	235 ± 25 (14)	203 ± 18 (16)
Overshoot (mV)	21.5 ± 1.9 (14)	18.7 ± 2.8 (14)	14.1 ± 1.9 (16)

Figures quoted are means ± s.e. mean. The number of fibres sampled is given in parentheses. *No. of fibres responding/No. of fibres tested. †A concentration of 1 μM was used in this experiment.

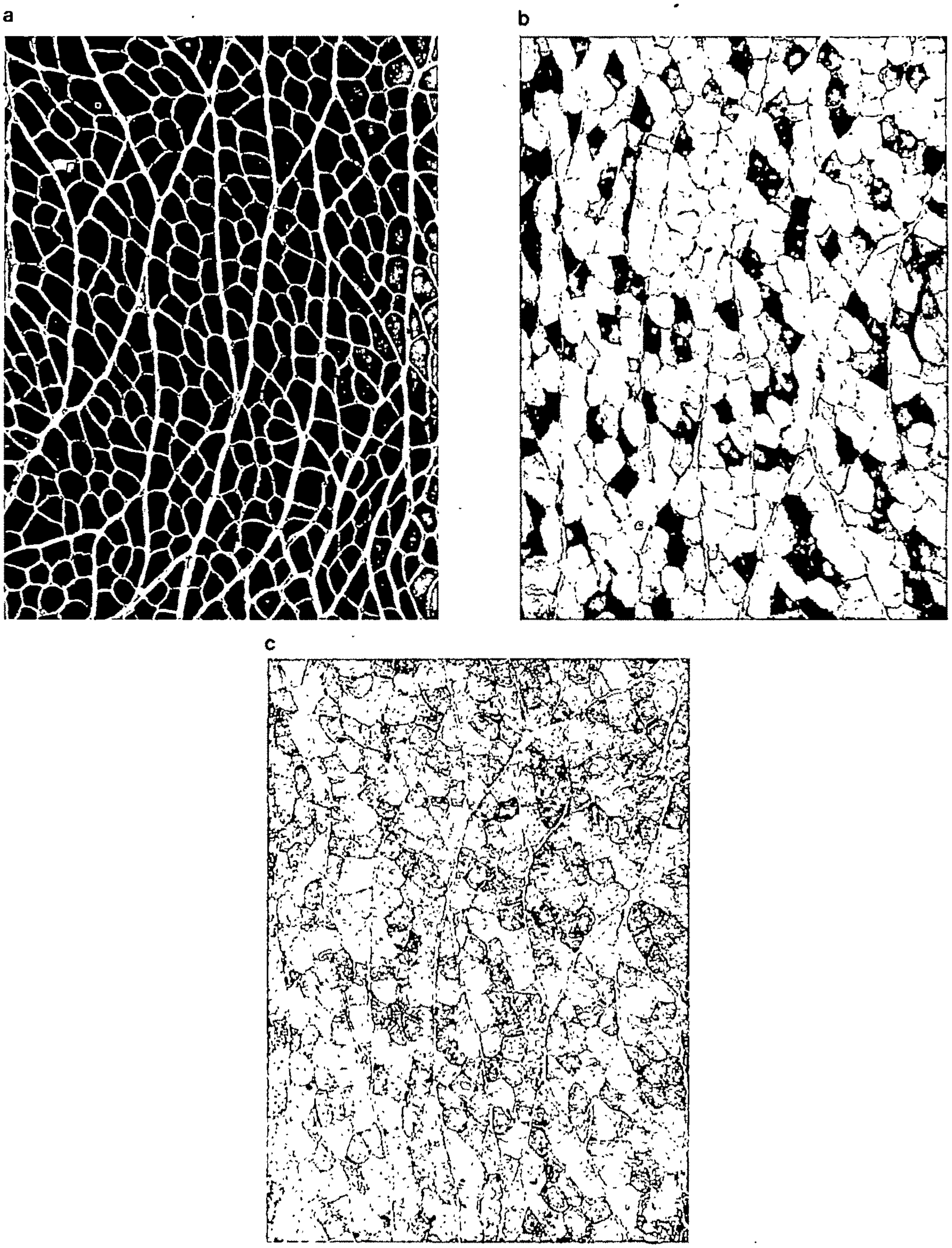


Figure 7 (a) Transverse section ×150 of part of an extensor digitorum longus muscle from an emetine (0.5 mg/kg)-treated rat stained with haematoxylin and eosin. Note the normal histological appearance of this muscle. (b) Transverse section ×150 serial to (a), stained with myofibrillar ATPase. Numerous areas of myofibrillar ATPase loss (pale areas in fibres) are seen, even though from haematoxylin and eosin staining in (a) the fibres are histologically normal. (c) Transverse section × 150 serial to (a) and (b) stained with NADH-TR. Foci of enzyme loss are seen in this preparation (pale areas in fibres) although the areas of loss are less well defined than in the myofibrillar ATPase preparation (b).

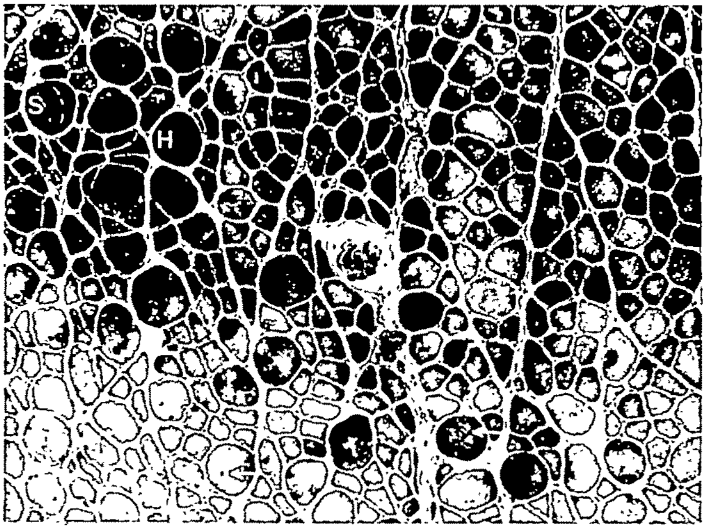


Figure 8 Transverse section ×150 of part of an extensor digitorum longus muscle from an emetine (2.0 mg/kg)-treated rat, stained with haematoxylin and eosin. Hyaline fibres (H) centrally nucleated fibres (arrows) and splitting fibres (S) can be seen.

Histology and histochemistry

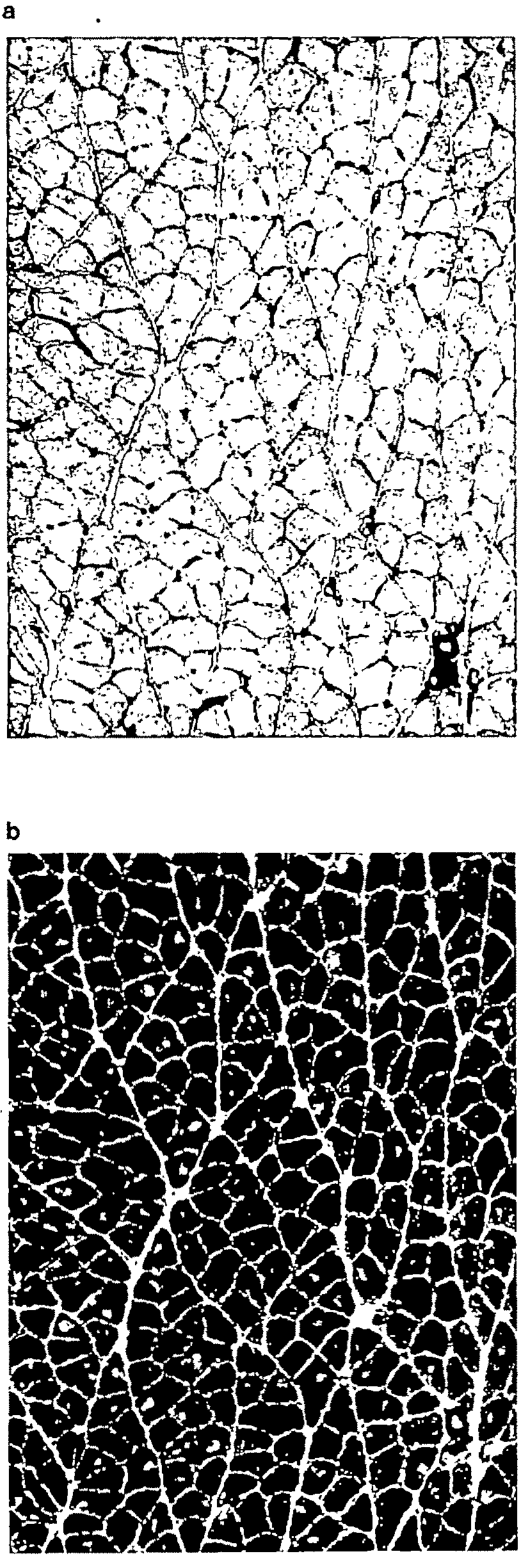
The chronic administration of low doses of (—)-emetine (0.5 mg/kg 5 days weekly for 180–220 days) had little effect on the histology or either EDL or SOL. There was no atrophy or loss of muscle fibres although an occasional necrotic fibre was seen in a few of the muscles studied. In less than 10% of the muscles studied, hyaline fibres (Bethlem, 1970) were seen but even in these muscles, the incidence of hyaline fibres was very low (< 1% of all fibres). There was also some evidence of fibre splitting and central nucleation.

Muscle fibres in EDL muscles which appeared normal when stained with haematoxylin and eosin often revealed a local loss of myofibrillar ATPase (Figure 8). This was a variable phenomenon. Thus of 12 EDL muscles studied, 4 were unaffected, 3 were severely affected and in the remaining 5, the changes were moderate. In the severely affected muscles up to 25% of the fibres were affected; in the least affected less than 5% of the fibres were affected. The reduction in ATPase activity was seen in both Type II and Type III fibres; since Type I fibres have low myofibrillar ATPase activity any further reduction in activity caused by (—)-emetine would be difficult to detect by the standard histochemical techniques. However, the acid-preincubation method of Brooke & Kaiser (1969) results in a 'reversal' of the ATPase staining characteristics of the main muscle fibre types. Thus, Type I fibres appear darkly stained in comparison to Type II and III, and the technique can therefore be used to detect any focal loss of ATPase activity in such fibres. However, no such focal loss of ATPase was seen in the Type I fibres of SOL, and only rarely was the phenomenon observed in the Type I fibres of EDL.

Type II and Type III fibres appeared to be equally susceptible to the focal loss of myofibrillar ATPase. In many fibres only one such area of reduced myofibrillar ATPase activity was seen, but as many as 3 such areas in a single fibre were not uncommon. The edges of these areas were not well defined, and suggested less severe disruption of myofibrillar material at the periphery (Figure 8). It is of interest to note that serial sections demonstrating the activity of NADH-TR revealed corresponding areas where the activity of this mitochondrial enzyme was also reduced (Figure 7). The loss in activity of NADH-TR appeared to be less severe than that of myofibrillar ATPase.

The demonstration of total myophosphorylase activity showed that this was virtually normal, except in the areas of total myofibrillar ATPase loss, and there was no evidence that the activity of either acid phosphatase or any other lysosomal hydrolytic enzyme was changed in the areas of myofibrillar ATPase loss.

Almost all of the animals receiving (—)-emetine over long periods of time showed loss of Type III fibres from SOL. This loss was not due to a selective atrophy of Type III fibres, but appeared in conjunction with an increase in the proportion of



'intermediate' fibres, suggesting that over the course of the experiment there was a transition of Type III fibres to Type I. However, control experiments (Johnson, unpublished) revealed that transition of Type III fibres to Type I fibres in (—)-emetine-treated SOL muscles was an index of maturation, rather than being drug-induced.

The systematic administration of larger doses of (—)-emetine (2.0 mg/kg) gave rise to similar but more severe changes as those previously described. In both SOL and EDL, hyaline fibres and splitting fibres were commonly seen (Figure 8). Similarly, the muscles with apparently normal histology as depicted by haematoxylin and eosin showed loss of myofibrillar ATPase activity and local loss of NADH-TR activity. However, unlike muscles from animals treated with low doses of (—)-emetine there was also a loss of myofibrillar ATPase in Type I fibres (demonstrated by the ATPase reversal technique; Figure 9) in the muscles of animals receiving these high doses of (—)-emetine.

Muscles from these animals were atrophied. The average wet weight of the EDL muscles for example fell from a normal value of 120 ± 3.5 mg ($n = 14$) to 97 ± 7.6 mg ($n = 6$).

Discussion

An attempt has been made to determine some of the features of (—)-emetine-induced myopathy in the rat. The study is not simply of academic interest since (—)-emetine is still occasionally used in the treatment of severe amoebiasis even though metronidazole is probably the drug of choice (Manson-Bahr & Ormerod, 1971). The highest dose we used in this study (2.0 mg/kg daily for 5 days in every 7) is not excessive by clinical standards; the dose of (—)-emetine used clinically is 60 mg/adult by injection daily for 10 days or more, often followed by chloroquine (which may also be myotoxic; Aguayo & Hudgson, 1970). This recommended dose, in the average adult, approximates to 1.0 mg/kg, and it is given by intramuscular or subcutaneous injection.

Generally, we found morphological and physiological evidence of muscle damage without any sign of involvement of the peripheral nervous system. Thus, even in animals receiving the highest doses of

Figure 9 (a) Transverse section ×150 of part of a soleus muscle from an emetine (2.0 mg/kg)-treated rat stained for myofibrillar ATPase. Foci of enzyme loss (pale areas) are seen in many of the Type I muscle fibres in this muscle. (b) Transverse section ×150 serial to (a) stained for myofibrillar ATPase after pre-incubation with formalin at pH 4.5. Type I fibres appear dark in this preparation, and the foci of enzyme loss are more easily distinguished.

the drug, neuromuscular function, as judged by the presence of miniature end-plate potentials and normal EMG records, was not impaired. Moreover, the muscles themselves exhibited none of the features associated with 'denervation' (see also Duane & Engel, 1970) and this lack of 'denervation-like changes' was not an indirect effect reflecting the ability of (—)-emetine to inhibit protein synthesis. The physiological changes that were demonstrated (i.e. fall in resting membrane potential, reduction in maximum rate of rise of the action potential) were more severe in the animals receiving high doses of (—)-emetine for 21–30 days than in those receiving lower doses for much longer periods. However, the precise significance of these changes is not clear since they occur in so many pathological conditions, involving directly or indirectly either peripheral nerve, skeletal muscle or both (see for example Harris, 1971; Albuquerque, Warnick, Tasse & Sansone, 1972; Harris & Ward, 1974) that they must be considered non-specific. The large increase in threshold for action potential generation seen in the muscles of the more severely affected animals may be of some significance. The threshold was shifted by 10 mV, from −50 mV in muscles from normal animals to approximately −40 mV in mucles from animals treated with 2.0 mg/kg (—)-emetine and this could have the effect of reducing the safety factor for neuromuscular transmission.

However, such a postulated reduction in safety factor cannot possibly account for the degree of weakness observed in these muscles since the muscles are weaker than normal when stimulated directly, and when their strength is expressed in terms of their individual wet weights. This latter observation would imply a direct involvement of the contractile material and/or its associated enzymes.

Our physiological and pharmacological observations of the properties of muscles removed from (—)-emetine-treated rats suggest to us that the demonstrable muscle weakness is primarily mediated by drug-induced damage to the muscle fibre rather than to an effect involving neuromuscular transmission. This conclusion is in direct contrast to that reached by Ng (1966) and Salako (1970). It is difficult to reconcile our differing points of view, particularly when it is considered that both Ng (1966) and Salako (1970) mainly described the acute effects of (—)-emetine administration to isolated or *in situ* preparations.

The observation that is most difficult to reconcile with our own is that of Salako (1970), who reported that following the injection of 1 mg/kg (—)-emetine subcutaneously the rat showed 'no spontaneous muscular activity' 'felt limp' and 'did not right itself when placed on its back'. We have never observed such a response, and have no explanation for this behaviour.

Salako (1970) also reported that rat sciatic nerve-gastrocnemius preparations, made *in vivo* in rats that had received a series of injections of emetine, had smaller contractions than normal and fatigued more rapidly than normal. This observation is not inconsistent with our findings, however, since Salako's results could be a demonstration of the inability of the muscle to generate tension, rather than a manifestation of partial neuromuscular failure induced by the prolonged administration of (—)-emetine.

Similarly, direct evidence of muscle damage was the observation of a dose-dependent focal loss of myosin ATPase suggesting a localized disruption of myosin filaments in (—)-emetine-damaged muscles. Preliminary observations by our colleagues (Bindoff & Cullen, personal communication) on the ultrastructural changes induced by emetine reveal such a disruption at least in Type II fibres. Similarly, the reduction of NADH-TR activity demonstrated by histochemistry is suggestive of mitochondrial damage, and autolytic changes involving the mitochondria have been seen by Bindoff & Cullen in both Type I and Type II fibres. In this context it is of interest that in the liver, (—)-emetine has been found to be associated with the mitochondria (Miller & Jondorf, 1970). Further, in the cardiac muscle of (—)-emetine-treated dogs, Murphy, Bullock & Pearce (1971) have correlated reduced mitochondrial oxidative phosphorylation with structural damage to the mitochondria.

Clearly a major disruption of mitochondrial metabolism would lead to the rapid loss of the appropriate cell; thus it might be expected that the mitochondria-rich muscle fibres (i.e. Type I) would be more susceptible to the drug than the relatively less mitochondria-rich Type II and Type III fibres. Similarly, muscles such as SOL that contain almost exclusively mitochondria-rich fibres, might be expected to be more severely affected than muscles such as EDL which contain a mixture of mitochondria-rich and mitochondria-poor fibres. It should be noted though, that the precise degree of damage caused by the drug in the different types of muscle can only be determined by quantitative electron-microscopic techniques. Until this determination is made, the role of mitochondrial damage in (—)-emetine-induced muscle damage remains unclear.

In summary, our observations, physiological, pharmacological and histological, all lead to the conclusion that the primary cause of muscular weakness, seen during the prolonged use of (—)-emetine is a direct effect of (—)-emetine on the skeletal muscle fibres. We cannot deny that the acute administration of (—)-emetine either to the whole animal, or to isolated mammalian tissues and organs may reveal other direct effects of the drug on, for example, ganglionic and neuromuscular transmission. Neither can we deny the possibility that there may be a slight, barely measurable effect of (—)-emetine on the safety factor of neuromuscular transmission in treated animals. However, it seems unlikely that the drug seriously impairs neuromuscular transmission either by inducing a neuropathy (Young & Tudhope, 1926) or by affecting the neuromuscular junction directly (Ng, 1966; Salako, 1970). It would appear, therefore, that (—)-emetine myopathy is a condition caused by the direct effects of the drug on skeletal muscle fibres.

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HYPOTHERMIC ACTION OF CHLORPROMAZINE IN MONKEYS

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1 In the conscious monkey (*Macaca cyclopis*) intravenous administration of chlorpromazine 0.1–8.0 mg/kg produced a fall of rectal temperature of 0.3 to 3.5°C after a latency of 3.5 to 10.8 minutes. The course of hypothermia lasted from 125 to 600 min or more.

2 Direct injection of chlorpromazine 100–800 μg into the lateral or fourth cerebral ventricle produced a similar fall of 0.3 to 2.0°C but with shorter latency (2 to 3.5 min) and with a duration of 94 to 375 minutes.

3 Two distinct structures in the brain stem, namely, the preoptic anterior hypothalamus (POAH) and medulla oblongata, responded to direct injection of chlorpromazine 200 μg. The sensitivity was slightly higher in the POAH than in the medulla. Structures in between were not sensitive.

4 The results suggest that chlorpromazine works principally through the central nervous system, i.e. the POAH and medulla oblongata, to mediate its hypothermic effect.

Introduction

The hypothermic action of chlorpromazine has been well documented and much effort has been devoted toward delineating its mechanism and sites of action. Peripheral mechanisms including α-adrenoceptor blockade (Goodman & Gilman, 1970), decreased utilization of glucose and free fatty acid (Bonaccorsi, Garattini & Jori, 1964; Ghafghazi, Miya, Mennear & Chalmers, 1968) have been suggested. Systemic injection of chlorpromazine uniformly produced hypothermia in several species i.e. rats, adult mice, hamsters, guinea-pigs, ground squirrel, rabbits and dogs (L'Allemand, Brendel & Usinger, 1955; Bachtold & Pletscher, 1957; Chevillard, Giorno & Laury, 1958; Hoffman & Zarrow, 1958; Bagdon & Mann, 1965; Kirkpatrick & Lomax, 1971; Reigle & Wolf, 1971).

It has been suggested that chlorpromazine may mediate its hypothermic action through the central nervous system (CNS). Chlorpromazine inhibited the neuronal activities of the reticular formation of the brain stem (Bradley, Wolstencroft, Hosli & Avanzino, 1966). Chlorpromazine also inhibited the gamma motor neurone and the brain stem facilitatory mechanism which led subsequently to relaxation of the skeletal muscle (Domino, 1962). The highest concentration of chlorpromazine has been found in the brain of dogs after a systemic dose of this agent (Salzman & Brodie, 1956). However, experimental findings regarding chlorpromazine hypothermia induced by CNS activation are controversial. In hamsters, Reigle & Wolf (1971) found that microinjection of chlorpromazine into the preoptic anterior hypothalamus (POAH) produced hypothermia. In rats, Rewerski & Jori (1968), Rewerski & Gumulka (1969) and Kirkpatrick & Lomax (1971) reported that similar intrahypothalamic injection produced an opposite effect, i.e. hyperthermia, although systemic injection produced hypothermia.

The present investigation was designed to elucidate further the hypothermic action of chlorpromazine. The site of action of chlorpromazine hypothermia was determined by comparing the effects of this agent by various routes of administration. Emphasis was laid particularly on local injection of chlorpromazine to various sites of the brain stem from the POAH through to the medulla oblongata. An attempt was made not only to localize the structures responsible for the hypothermia but also to determine whether structures other than the POAH are sensitive to chemical agents which cause change of body temperature. Previous investigations of ours (Chai, Mu & Brobeck, 1965; Chai & Wang, 1970; Chai, Chen & Yin, 1971; Chai & Lin, 1972) and of others (Holmes, Newman & Wolstencroft, 1960) have shown that the medulla is thermosensitive and may participate in thermo-regulation. In the present investigation, monkeys were used because little information regarding the thermal effects of chlorpromazine on this species is available.

Methods

Thirty-eight monkeys (*Macaca cyclopis*) of either sex, weighing 4–5.5 kg were used. The animals were prepared for intracerebroventricular or intracerebral administration according to the methods of Chai *et al.* (1971) and Myers & Yaksh (1969) with some modifications. In brief, each animal was anaesthetized with sodium pentobarbitone (35 mg/kg, i.v.). Under aseptic procedures, a guide cannula, made of a 21-gauge lumbar puncture needle cut to the desired length, was positioned in the lateral ventricle or fourth ventricle, or in the cerebral tissue of the brain stem by means of a stereotaxic technique. The cannulae were fixed on the skull by stainless steel screws and acrylic resin. For intracerebral injection, usually 2–4 cannulae were implanted at random into the brain stem of the animal at a time. In the area rostral to midbrain the cannulae were inserted at a right angle while in the area caudal to mid-brain at a 50° angle. This was done in order to allow the cannula to be perpendicular to the base of the brain stem. After completion of the implantation, the central occluding stylus was inserted into the cannula and was fastened by threads.

The animals were not subjected to experimentation until at least 5 to 7 days after they had recovered completely from the surgery. When the intracerebral injection was given, the central occluding stylus was removed and an injection cannula, made of a 25-gauge tubing needle with the length cut 0.5 mm longer than the guide cannula, was inserted into the guide cannula. The free end of the needle tubing was connected to a section of PE 20 polyethylene tubing and the injection was given from a 100 µl Hamilton microsyringe. Intravenous administration was directly through the saphenous vein.

A solution of chlorpromazine hydrochloride (Rhdia, France) was prepared fresh before use by dissolving it in pyrogen-free 0.9% w/v NaCl solution (saline). For intravenous injection (i.v.) a solution was prepared in which 1 ml contained the per kg dose, 0.1–8 mg; for intraventricular injection 100 µl contained the total dose, 100–800 µg; for intracerebral injection, 10 µl contained 200 µg of the agent.

The pH of the chlorpromazine solution for intracerebroventricular injection was 5.3–5.7 with a tonicity equivalent to 0.91–1.03% NaCl solution. The chlorpromazine solution for intracerebral injection was at pH 5.0 with a tonicity equivalent to 1.23% NaCl solution. Control injections of the same volume of saline adjusted to the above range of acidity and tonicity via the same routes did not produce any significant response.

Twelve hours before experimentation the monkey was allowed no food, but water was available *ad libitum*. During the experiments the animal was restrained in a monkey chair at a constant environmental temperature of 25 ± 0.5°C. Injection of the drug was made only after the animal had acclimatized to the environment and the rectal temperature had become stable. Repeat injections were made at an interval of 4–6 days.

Rectal temperature was monitored by a flexible thermistor probe (Yellow Springs Instrument Co. (YSI) 401) inserted 8–10 cm into the rectum. Cutaneous temperature was monitored by a needle thermistor probe (YSI 524) inserted into the subcutaneous tissue on the inner side of the thigh, and by a surface thermistor probe (YSI 425) adhering to the surface of the palm. Temperature signals were amplified by a YSI scanning telethermometer (model 47). Respiratory rate was monitored by means of a pneumograph. All recordings were made on a polygraph (Grass 7B). An electrocardiogram, lead II, was recorded by a separate polygraph. Heart rate was estimated by counting the number of R waves of the ECG.

At the conclusion of each experiment, 10 µl of diluted China ink was injected into the guide cannula for labelling. The animal was killed by an overdose of sodium pentobarbitone and the head was perfused with saline, followed with 10% formalin solution. The brain was then removed, and the sites of injection were verified by means of frozen sections of 30 µm thickness stained by the Weil method.

Results

General reactions after administration of chlorpromazine

The overall reactions varied with the dose of chlorpromazine. When the dose was less than 0.1 mg/kg intravenously or below 100 µg intracerebroventricularly (lateral or 4th ventricle), no apparent changes in behaviour or body temperature were observed. At higher doses the animal exhibited struggling, restlessness, polypnoea and increase of the cutaneous temperature of the palm during the first 5–15 minutes. These responses were then followed by a period of sedation, during which there was evidence of decreased muscle tonus, with extension of the extremities, sleepy appearance, and sluggishness in the corneal reflex concomitant with a drop in body temperature. The degree of hypothermia was proportional to the extent of sedation. The size of the pupil remained essentially unchanged. Half to several hours after sedation the animal gradually regained full consciousness. Rectal temperature gradually returned as the sedation diminished; the animal often urinated at this time.

Effects of intravenous administration of chlorpromazine on body temperature

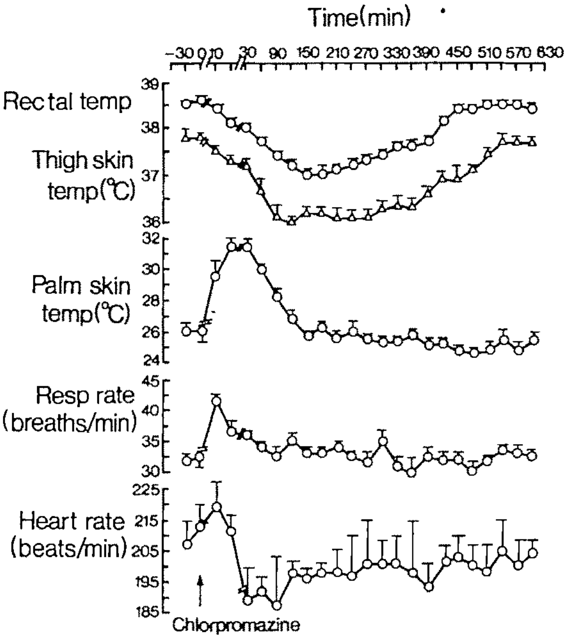

Figure 1 Changes of rectal temperature, cutaneous temperature of the thigh and palm, and respiratory and heart rates after chlorpromazine, 2 mg/kg intravenously. Administration of drug was performed at zero time. $n = 6$.

Table 1 summarizes the effects of chlorpromazine, 0.1–8 mg/kg, intravenously. In 3.5–10.8 min after the dose, the rectal temperature began to fall and reached a minimum in 40–200 minutes. The average fall in rectal temperature was 0.3–3.5°C, proportional to the dose. At a dose of 0.1 mg/kg to 4 mg/kg the temperature gradually returned to its preinjection level in 125–576 minutes. When the dose was increased to 8 mg/kg, the temperature did not return to its preinjection level even after over 600 minutes. Figure 1 shows the time course of the average change in six animals in rectal temperature, subcutaneous temperature of the thigh and cutaneous temperature of the palm, and respiratory and heart rates after chlorpromazine, 2 mg/kg, intravenously. The fall in rectal temperature (maximum 1.7°C) paralleled the fall of subcutaneous temperature of the thigh (maximum 1.8°C). The temperature reached a minimum in 150 min, then rose gradually to return to the preinjection level after 450 minutes. In contrast, after chlorpromazine injection, the cutaneous temperature of the palm rose very rapidly to 5.5°C higher than normal in 20 minutes. The rise, however, was maintained only briefly and temperature returned to the preinjection level in 150 minutes. Concomitant with the elevation of palm temperature, respiratory rates increased (9 breaths/min on average) but for only a short time (90 minutes). The time course of reduction of heart rate varied with different animals. In two out of six animals, a decrease occurred immediately after the chlorpromazine injection. In one

Table 1 Decrease of rectal temperature after intravenous and intracerebroventricular administration of chlorpromazine

Route of administration	*Dose*	*Latency (min)*	*Maximum fall in rectal temp. (°C)*	*Time to minimum temp. (min)*	*Time of recovery (min)*
Saphenous vein	0.1 mg/kg	10.8 ± 2.2	0.3 ± 0.1	40 ± 12	125 ± 34
	0.5 mg/kg	6.6 ± 0.8	1.2 ± 0.2	73 ± 16	234 ± 53
	1.0 mg/kg	6.2 ± 0.7	1.4 ± 0.4	102 ± 33	359 ± 83
	2.0 mg/kg	4.3 ± 0.6	1.7 ± 0.2	102 ± 10	429 ± 41
	4.0 mg/kg	4.5 ± 0.5	2.7 ± 0.2	179 ± 10	576 ± 14
	8.0 mg/kg	3.5 ± 0.3	3.5 ± 0.3	200 ± 53	*
Lateral Ventricle	100 μg	3.3 ± 0.6	0.4 ± 0.1	35 ± 10	144 ± 51
	200 μg	3.5 ± 0.4	1.2 ± 0.2	69 ± 4	168 ± 39
	400 μg	4.0 ± 0.7	1.7 ± 0.2	64 ± 8	314 ± 61
	800 μg	2.5 ± 0.4	1.9 ± 0.1	97 ± 10	229 ± 11
Fourth Ventricle	100 μg	3.5 ± 0.4	0.3 ± 0.1	24 ± 5	94 ± 23
	200 μg	3.0 ± 0.1	1.0 ± 0.1	56 ± 24	185 ± 19
	400 μg	3.2 ± 0.4	1.7 ± 0.1	75 ± 12	246 ± 51
	800 μg	2.0 ± 0.1	2.0 ± 0.1	100 ± 7	375 ± 95

Values are means ± s.e. of results from six animals.

* At this dose none of 6 animals returned to the preinjection temperature within 10 hours.

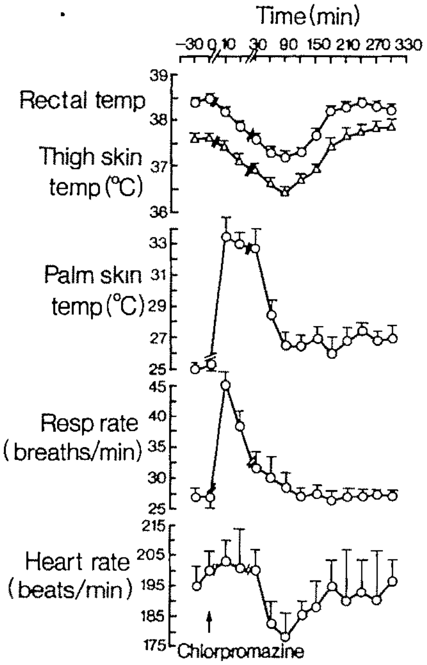

Figure 2 Changes of rectal temperature, cutaneous temperature of the thigh and palm, and respiratory and heart rates after chlorpromazine 200 μg injected into the lateral ventricle. $n = 6$.

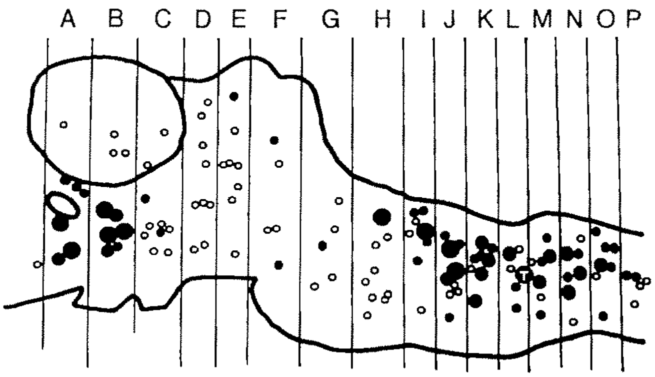

Figure 3 Sagittal section showing sites of intracerebral injection of chlorpromazine 200 μg. Areas of the brain stem are taken 1 mm from the midline. The closed circles show positive reactions and indicate that injections produced a hypothermic response greater than 0.5°C with a latency shorter than 5 minutes. The sizes of the circles illustrate the thermal reactivity: small, 0.5 to 0.8°C; medium, 0.8 to 1°C, large, above 1°C. The open circles indicate negative results, showing that either the decrease of rectal temperature was less than 0.5°C or the latency of onset longer than 5 min, or both. A = preoptic anterior hypothalamus, B = middle hypothalamus, C = posterior hypothalamus, D and E = midbrain, F to I = pons, J to P = medulla.

animal the heart rate did not show any change in the first 20 min but then decreased gradually. In three other animals the heart rate showed an initial elevation and then decreased gradually in about 30 minutes. The mean maximum decrease in heart rate was 20 beats/minute. In general, the decrease of heart rate lasted for a relatively long period (300 min average) and was frequently accompanied by changes of electrocardiogram, i.e. irregular P–R interval, S–T depression, or elevation of T wave.

Effects of intracerebroventricular administration of chlorpromazine on body temperature

Table 1 also summarizes the fall of rectal temperature after administration of chlorpromazine 100–800 μg to the lateral or fourth ventricle. The hypothermic responses from these two routes were similar. The magnitude of temperature reduction was dose-related, in a range of 0.3–2°C; the latency of onset 2.0–3.5 min; the duration of maximum reaction 24–100 min; the whole course of the hypothermic reaction 94 to 375 minutes. Figure 2 shows the result of administration of chlorpromazine 200 μg through the lateral ventricle. In general, the reactions followed the same patterns as those of intravenous and intracerebral injections. After chlorpromazine the temperature of rectum and thigh fell 1.2°C in 90 minutes. The palm temperature and respiratory rate showed a rapid but brief increase, 8.2°C and 18 breaths/min, 10 to 20 min after the injection. After chlorpromazine, the heart rate showed a slight increase and a decrease (22 beats/min) 30 min later. Cardiac arrhythmia also occurred frequently. The time course and pattern of reactions following administration of chlorpromazine into the 4th ventricle were similar.

Effects of intracerebral administration of chlorpromazine on body temperature

Figure 3 shows the sites of local administration of chlorpromazine 200 μg into the brain stem. The reactive sites were found to be confined principally to two areas i.e. the POAH and medulla oblongata. Other areas such as posterior hypothalamus, midbrain, and pons were not sensitive. Table 2 summarizes the results of intracerebral injection of chlorpromazine 200 μg. A total of 120 injections were made. Among the 17 sites of injection in the POAH, 12 produced a positive reaction, of which 5 showed a hypothermic response more than 1°C (the greatest being, 1.8°C). Only 2 of the 10 injections in the posterior hypothalamus showed positive reactions; one of the 17 injections in the midbrain did so. Four of 19 injections in the pons produced a hypothermic response. One of these points produced a fall of more than 1°C. Among the 57 injections in the medulla, 40 showed a hypothermic response, and 4 of these produced hypothermia of more than 1°C (highest 1.5°C). If the degree of sensitivity among the various regions of the brain stem is expressed as the 'effective hypothermic index' (the extent of rectal temperature fall times the percentage of positive reactions in a particular brain area), the sensitivity is in the following descending order: POAH (0.71), medulla (0.59), pons (0.13), posterior hypothalamus (0.12), midbrain (0.04). Figure 4 shows the results of local injection of chlorpromazine 200 μg into the medulla (labelled T in Figure 3L). The pattern of reactions did not differ from those of other routes of injection. The maximum fall in rectal temperature was 1.1°C, along with a fall of thigh subcutaneous temperature (maximum 0.8°C). Palm temperature rose 5°C. Respiratory rate increased 10 breaths/minute. Heart rate decreased 27 beats/minute.

On two occasions during medullary injections, the

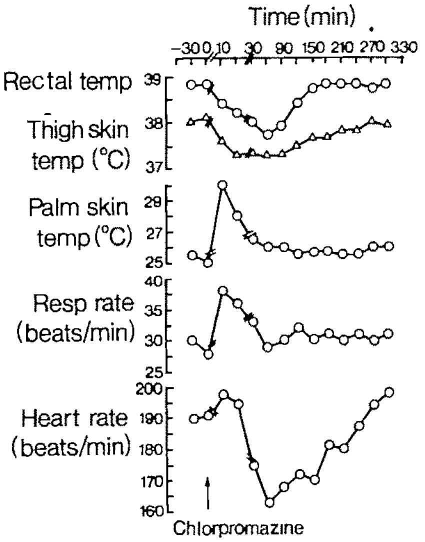

Figure 4 Changes of rectal temperature, cutaneous temperature of the thigh and palm, and respiratory and heart rates after chlorpromazine 200 μg injected into the medulla oblongata (obtained from the 'T' point in L of Figure 3). $n = 1$.

Table 2 Results of local injection of chlorpromazine (200 μg) into preoptic anterior hypothalamus, posterior hypothalamus, midbrain, pons and medulla oblongata.

	Preoptic anterior hypothalamus	*Posterior hypothalamus*	*Midbrain*	*Pons*	*Medulla*
No. of injection sites	17	10	17	19	57
No. of injection sites showing hypothermic response	12	2	1	4	40
No. of injection sites showing negative response	5	8	16	15	17
Average of fall in rectal temperature (°C)	1.0	0.6	0.7	0.6	0.8
Effective percentage	70.6%	20.0%	5.9%	21.0%	70.2%
Effective hypothermic index	0.71	0.12	0.04	0.13	0.56

Hypothermic and negative responses are explained in the text.

$$\text{Effective percentage} = \frac{\text{No. of injection sites showing hypothermic response}}{\text{No. of injection sites}} \times 100.$$

Effective hypothermic index = Average fall of rectal temperature × effective percentage.

rectal temperature, after an initial fall for periods of 155 and 180 min, rose to a level of 0.9°C and 1.5°C higher than the normal temperature, as previously reported by Chai *et al.* (1971).

Discussion

The findings show that intracerebral administration of chlorpromazine is effective in producing hypothermia. Injection of chlorpromazine 200 µg, both into the lateral and 4th ventricles produced a fall of rectal temperature from 1°C to 1.2°C, comparable to that following chlorpromazine 0.5–1 mg/kg intravenously (Table 1). This dose was 1/15 to 1/25 that of the intravenous injection (each animal weighed 5 kg on average). In some animals, an even smaller dose (1/100 that of i.v.) was sufficient. In addition, the latency of hypothermia after intracerebroventricular injection was much shorter than when the drug was administered by the intravenous route. This suggests that the hypothermic effect of chlorpromazine was in great part mediated through the CNS. Similar results have been observed in the rat (unpublished data). In this investigation, administration of chlorpromazine into the 3rd and 4th ventricles, and systemic circulation all produced hypothermia. Administration through the ventricle required a much smaller dose yet produced a greater effect. It should be noted, however, that intravenous administration of a large dose of chlorpromazine produced a greater hypothermia than that of the cerebroventricular route; 3.5°C after 8 mg/kg intravenously compared with 2°C after 800 µg intracerebroventricularly (see Table 1). A peripheral component in the hypothermic action cannot be excluded.

It is generally agreed that in the POAH there resides the highest integrating mechanism of thermohomeostasis. Direct injection of pyrogen into this area induces fever (Cooper, Cranston & Honour, 1967; Lin & Chai, 1972). Injection of morphine (Lotti, Lomax & George, 1965), acetylcholine (Kirkpatrick & Lomax, 1970), or catecholamine (Myers & Yaksh, 1969) also produce very marked changes in the body temperature. However, current investigations have shown that regulation of body temperature is not limited to POAH. For example, Hardy (1973) has recorded input signals in the anterior and posterior hypothalamus and midbrain when the skin, hypothalamus or spinal cord were heated or cooled. We also have repeatedly demonstrated that cooling of the medulla oblongata and spinal cord produced subcutaneous vasoconstriction, slowing of respiratory rate, tachycardia, hypertension and increase of rectal temperature while heating produced the opposite effects, i.e., subcutaneous vasodilatation, respiratory acceleration, bradycardia, hypotension and decrease of rectal temperature (Chai *et al.*, 1965; Chai & Wang, 1970). These reactions persisted without reduction after decerebration or destruction of the POAH (Chai & Lin, 1973; Lin & Chai, 1974). Similar findings on the independence of thermosensitivity in the medulla have been reported by Lipton (1973). Rosendroff & Mooney (1971) have succeeded in inducing fever by local injection of leukocytic pyrogen not only in the hypothalamus but also in the pontine reticular formation. However, they did not explore the medulla oblongata. On the other hand, Bard, Woods & Bleir (1970) observed that chronic decerebrate cats failed to produce any effective defence actions during cold exposure and no fever response after administration of pyrogen. Cooper *et al.* (1967) injected leukocytic pyrogen into various parts of the brain and found that only injection in the POAH produced fever. Other areas, i.e. posterior hypothalamus, midbrain and pons were not responsive. It should be noted that there were few injections to areas caudal to the posterior hypothalamus.

In the present experiments, we injected chlorpromazine into the whole brain stem of monkeys and found that two distinct structures, i.e. the POAH and medulla, with the former predominant, were very sensitive in hypothermic reaction. The areas are identical with those responding to local injections of acetylstrophanthidin for hypothermia as found previously by Chai *et al.* (1971). Acetylstrophanthidin probably produces hypothermia by its acetylcholine-like action. It has been found that acetylcholine produces hypothermia when injected into the hypothalamus (Kirkpatrick & Lomax, 1970).

The above results thus are consistent with the contention that the POAH plays a very important role in temperature regulation, and that an independent but accessory mechanism for regulation exists in the medulla oblongata (Chai & Lin, 1973; Lin & Chai, 1974).

Thermoregulatory reactions, i.e., cutaneous vasodilatation of the palm, increased cutaneous temperature and respiratory acceleration were apparent shortly after the administration of chlorpromazine. It should be noted that vasodilatation occurred only on the palm and foot but not on the thigh. Since the skin of the palm is not covered with hair, this may suggest that heat dissipation is achieved principally through redistribution of more heat load to the hairless region. It should be noted that both vasodilatation and respiratory acceleration are important mechanisms for heat dissipation. Thus it appears very likely that increase of heat dissipation is the chief mechanism of the chlorpromazine-induced hypothermia, at least in the early stage after administration of the drug.

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EFFECTS OF SOME PURINE DERIVATIVES ON THE GUINEA-PIG TRACHEA AND THEIR INTERACTION WITH DRUGS THAT BLOCK ADENOSINE UPTAKE

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1 Adenosine, adenosine 5′-triphosphate (ATP), adenine, inosine and guanosine all caused concentration-dependent relaxations of guinea-pig tracheal smooth muscle *in vitro*. The relative potencies in descending order were: adenine ⩾ guanosine > inosine ⩾ adenosine ⩾ ATP

2 Responses to the purine compounds were unaffected by propranolol (1 μg/ml).

3 The spasmolytic potencies of adenosine and ATP were greatly enhanced in the presence of the adenosine uptake blocking drugs dipyridamole, hexobendine or Dilazep, whereas responses to adenine were unaffected and those to inosine and guanosine were reduced.

4 The spasmolytic potencies of noradrenaline, aminophylline, prostaglandin E_2 and glyceryl trinitrate were unaffected by dipyridamole, hexobendine and Dilazep.

5 It is suggested that an adenosine uptake process may exist in the trachea of the guinea-pig and that this process is inhibited by dipyridamole, hexobendine and Dilazep.

Introduction

Coleman & Levy (1974) demonstrated the spasmolytic activity of adenosine and adenosine 5′-triphosphate (ATP) in the guinea-pig isolated tracheal tube preparation. This spasmolytic activity was enhanced by three drugs reported to inhibit adenosine uptake, dipyridamole (Kolassa, Pfleger & Rummel, 1970), hexobendine (Kraupp, Wolner, Adler-Kastner, Chirikdjian, Ploszczanski & Tuisl, 1966) and Dilazep (Sano, Katsuki & Kawada, 1972; Buyniski, Losada, Bierwagen & Gardier, 1972). The present work is a more detailed study of spasmolytic activity in a range of purine derivatives on the guinea-pig isolated tracheal tube preparation and of the effects of drugs that block adenosine uptake on this activity.

Methods

Guinea-pigs weighing 300–400 g were killed by a blow on the head and the tracheas excised and set up in a manner similar to that described by Farmer & Coleman (1970). Two tracheal tube preparations were made from each animal by dividing the trachea halfway along its length. Each portion was mounted on a tracheal holder (Figure 1), modified from that described by Farmer & Coleman (1970). A high level of resting tone was induced in the tracheal tube as

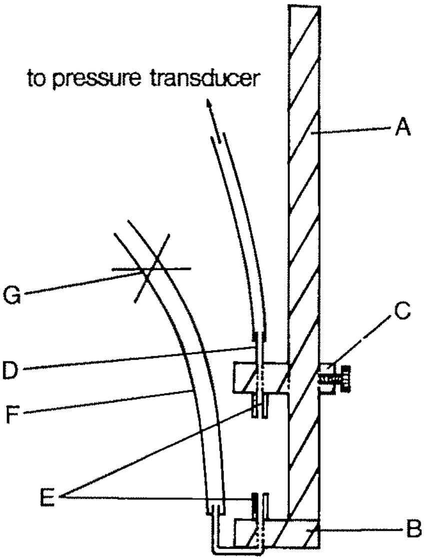

Figure 1 Diagram of tracheal holder. A: perspex rod. B: foot. C: adjustable collar. D: stainless steel tube (0.055 inch diam., 17 G). E: silicon rubber sleeve. F: silicon rubber tube. G: clip.

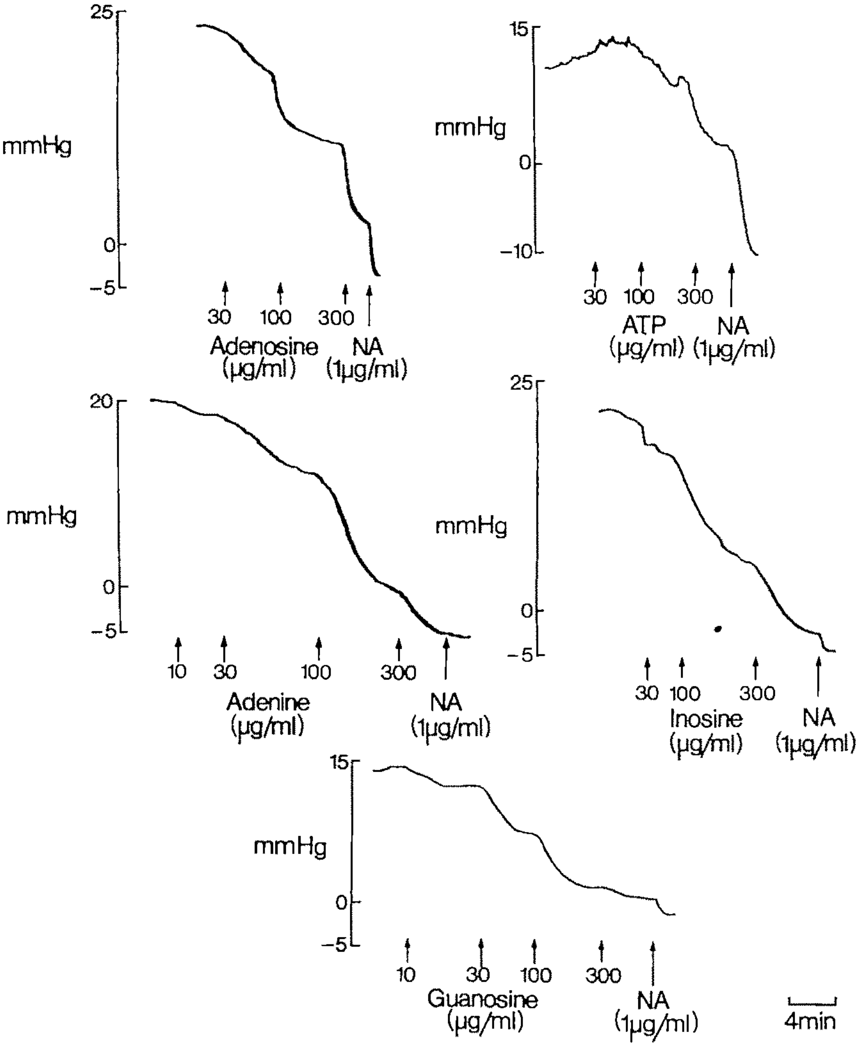

Figure 2 Guinea-pig isolated tracheal tube. Cumulative effects of adenosine, adenosine 5′-triphosphate (ATP), adenine, inosine and guanosine in relaxing spontaneous tone. NA = noradrenaline.

described by Coleman & Farmer (1971) and the intraluminal pressure (1 mmHg ≈ 133 Pa) was monitored continuously. Physiological salt solution maintained at 37°C was in contact with both inner and outer surfaces of the trachea. It had the following composition (g/l): NaCl 6.9, KCl 0.35, KH_2PO_4 0.16, $MgSO_4.7H_2O$ 0.29, glucose 2.0, $NaHCO_3$ 2.1, $CaCl_2.6H_2O$ 0.28.

Cumulative concentration-effect curves to spasmolytic drugs were determined. On completion of each curve a maximally effective concentration of noradrenaline (1 µg/ml) was added to the bathing solution to determine the 100% spasmolytic response.

In experiments in which the effects of drugs that block adenosine uptake were investigated, concentration-effect curves for the spasmolytic agents alone were repeated until constant results were obtained; the uptake blocking drug was then added to the bathing solution and the responses to the spasmolytic agents redetermined. Concentration-effect curves for the spasmolytic agents were repeated in the presence of the uptake blocking drug until any change in sensitivity became maximal. Spasmolytic responses to inosine, guanosine and prostaglandin E_2 were found to cause tachyphylaxis and so experiments were carried out on paired preparations, one half serving as untreated control and the other being exposed to a drug blocking adenosine uptake.

Concentration-ratios were calculated from EC_{40} values before and after addition of the uptake blocking drug. EC_{40} values were used because 50% inhibition of spontaneous tone was not achieved in every experiment.

Drugs

The following drugs were used: adenine (Boehringer Mannheim); adenosine (Koch-Light); adenosine 5′-triphosphate (ATP, BDH); aminophylline (A & H); tetrahydro-1H-1, 4-diazepine-1,4 (5H)-dipropanol 3,4,5-trimethoxybenzoate (diester) (Dilazep, Asta Werke); dipyridamole (Boehringer Ingelheim); glyceryl trinitrate (BDH); guanosine (Koch-Light); hexobendine (Oestereichische Stickstoffwerke); inosine (Koch-Light); (–)-noradrenaline bitartrate (Winthrop); propranolol hydrochloride (ICI); prostaglandin E_2 (Cambrian).

Results

Spasmolytic activity of purine derivatives

All the purines tested produced concentration-dependent spasmolytic responses in isolated tracheas (Figure 2) within the concentration range 10–300 µg/ml. Frequently ATP (30 µg/ml) and occasionally adenosine (30 µg/ml) elicited excitatory responses as previously reported (Coleman & Levy, 1974) at concentrations lower than those required for inhibition.

Adenine consistently caused 90–100% inhibition of spontaneous tone at the highest concentration (300 µg/ml) but none of the other purines achieved maximal inhibition of tone in concentrations up to 300 µg/ml, this being the practical limit of their solubilities. The responses to inosine and guanosine often caused tachyphylaxis but the concentration-effect curves for adenosine, ATP and adenine were reproducible. The order of spasmolytic potency over

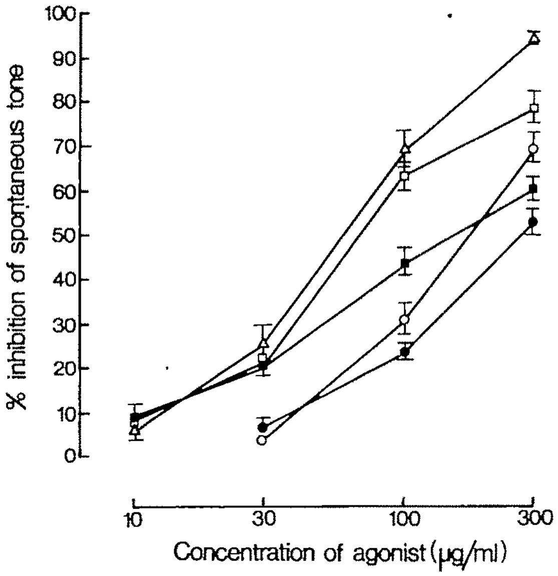

Figure 3 Guinea-pig isolated tracheal tube. Cumulative concentration-effect curves for the inhibition of spontaneous tone by adenosine (○), adenosine 5′-triphosphate (●), adenine (△), inosine (■) and guanosine (□). Each point represents the mean of at least 12 experiments and vertical bars indicate s.e. mean.

Table 1 The effect of dipyridamole, hexobendine and Dilazep on concentration-effect curves to adenosine in the guinea-pig isolated tracheal tube

Compound	*Concentration µg/ml*	*No. of experiments*	*Potentiation ratio* $\frac{EC_{40}\ control}{EC_{40}\ treated}$	*95% Confidence limits*
Dipyridamole	0.1	4	9.9	2.8– 35.1
	1	8	44.5	23.8– 83.3
	10	8	123.2	59.4–255.6
Hexobendine	0.1	6	9.8	2.5– 38.8
	1	6	20.4	13.6– 30.6
	3	4	50.6	40.1– 63.8
	10	6	14.3	8.6– 23.7
Dilazep	0.1	5	26.8	12.9– 55.4
	1	5	29.8	16.4– 54.1
	10	5	33.8	22.5– 50.8

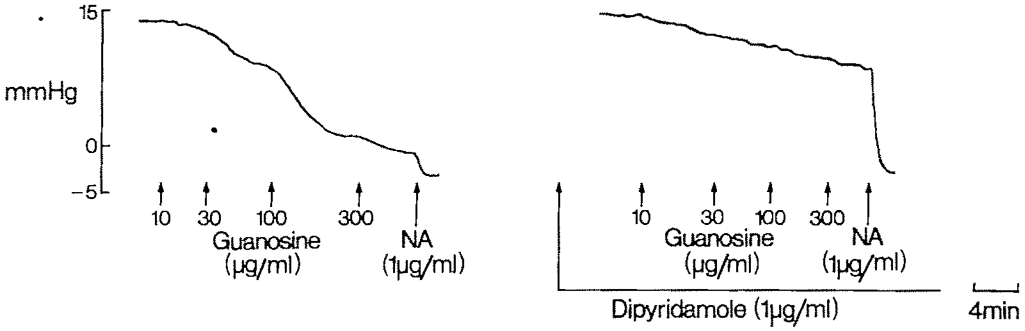

Figure 4 Guinea-pig isolated tracheal tube. Effect of dipyridamole (1 μg/ml) on cumulative effects of guanosine in relaxing spontaneous tone. NA = noradrenaline.

the whole series of experiments was adenine ⩾ guanosine > inosine ⩾ adenosine ⩾ ATP (Figure 3).

Effect of propranolol on responses to purine derivatives

The β-adrenoceptor antagonist propranolol (1 μg/ml), had no effect on the spasmolytic potency of the purine derivatives ($n = 4-8$).

Effects of dipyridamole, hexobendine and Dilazep on responses to adenosine

The spasmolytic potency of adenosine was clearly potentiated by all three drugs blocking adenosine uptake (Table 1). The potentiating effect of dipyridamole (0.1–10 μg/ml) was concentration-dependent as was that of hexobendine up to 3 μg/ml, but hexobendine was less active at 10 μg/ml than at 3 μg/ml. The potentiating effect of Dilazep was virtually the same over the range tested (0.1–10 μg/ml).

Effects of dipyridamole, hexobendine and Dilazep on responses to ATP, adenine, inosine and guanosine

The concentrations of drugs blocking adenosine uptake used in these experiments had been shown (Table 1) to potentiate the action of adenosine. The effects of these agents on the responses to the other purines are summarized in Table 2.

The effects of ATP were potentiated in much the

Table 2 The effect of dipyridamole (1 μg/ml), hexobendine (3 μg/ml) and Dilazep (1 μg/ml) on concentration-effect curves to adenosine 5′-triphosphate (ATP), adenosine, inosine and guanosine in the guinea-pig isolated tracheal tube

Purine derivative	*Treatment*	*No. of experiments*	*Potentiation ratio* $\frac{EC_{40}\ control}{EC_{40}\ treated}$	*95% Confidence limits*
ATP	Dipyridamole	4	63.3	28.8–139.1
	Hexobendine	4	55.7	40.4– 76.8
	Dilazep	6	22.7	12.9– 39.9
Adenine	Dipyridamole	4	1.5	1.1– 2.1
	Hexobendine	4	1.3	0.8– 2.3
	Dilazep	4	1.4	0.7– 2.7
Inosine	Dipyridamole	4	<1	–
	Hexobendine	3	<1	–
	Dilazep	4	<1	–
Guanosine	Dipyridamole	6	<1	–
	Hexobendine	4	<1	–
	Dilazep	4	<1	–

same way as those of adenosine but the spasmolytic potency of adenine was not much affected by the adenosine uptake blocking drugs. Surprisingly, the responses to both inosine and guanosine were reduced but the effect was difficult to quantify because the maximal response to each drug was reduced in the presence of the uptake blocking agents. A typical experiment showing the interaction between guanosine and dipyridamole is illustrated in Figure 4.

Effects of dipyridamole, hexobendine and Dilazep on responses to other spasmolytic drugs

The effects of the three drugs blocking adenosine uptake on the spasmolytic potencies of noradrenaline, aminophylline, prostaglandin E_2 and glyceryl trinitrate are summarized in Table 3. The only significant potentiation was a modest 2.4-fold increase in the activity of glyceryl trinitrate in the presence of dipyridamole.

Discussion

Adenosine and certain related compounds cause relaxation of the smooth muscle in a number of structures including that in the gastrointestinal tract (Burnstock, 1972) and the coronary vasculature (Berne, 1964), and tracheal bronchial smooth muscle (Bennett & Drury, 1931; Florey & Wells, 1931; Lendle, 1937; Meves, 1953; Bianchi, de Natale & Giaquinto, 1963; Bertelli, Bianchi & Beani, 1973). Some of these effects of adenosine are potentiated by drugs such as dipyridamole, hexobendine and Dilazep which were originally developed as coronary vasodilators. All of these compounds have been reported to potentiate the effect of adenosine on the coronary vasculature (Kraupp, Heistracher, Wolner & Tuisl, 1964; Hilger, 1969; Kolassa *et al.*, 1970; Buyniski *et al.*, 1972; Sano *et al.*, 1972) and dipyridamole and hexobendine to potentiate the negative chronotropic effect of adenosine on cardiac muscle (Hockerts & Bögelmann, 1959; Hopkins & Goldie, 1971; Kolassa, Pfleger & Träm, 1971). The mechanism of the potentiation of adenosine by these agents may involve inhibition of adenosine uptake since Pfleger, Volkmer & Kolassa (1969) and Hopkins (1973) have shown that dipyridamole and hexobendine inhibit the uptake of [^{14}C]-adenosine into guinea-pig hearts. However, not all of the effects of adenosine are potentiated by drugs of this kind. For example, they do not potentiate adenosine-induced relaxation of rabbit gastro-intestinal smooth muscle (Stafford, 1966; Hulme & Weston, 1974) or portal vein (Hughes & Vane, 1970). Presumably, the adenosine uptake process is present in some but not all tissues.

All the purine derivatives employed in this investigation relaxed guinea-pig tracheal smooth muscle by a mechanism not involving stimulation of β-adrenoceptors. These agents were about 1,000 times less active than noradrenaline. Furthermore, the potentiation of adenosine responses in the trachea by the uptake blocking drugs suggests that an uptake process for this nucleoside, similar to that present in the heart and coronary vasculature may also exist in the trachea.

The spasmolytic potency of ATP was similar to

Table 3 The effect of dipyridamole (1 µg/ml), hexobendine (3 µg/ml) and Dilazep (1 µg/ml) on concentration-effect curves to noradrenaline, aminophylline, prostaglandin E_2 and glyceryl trinitrate in the guinea-pig isolated tracheal tube

	Treatment	*No. of experiments*	*Potentiation ratio* $\frac{EC_{40}\ control}{EC_{40}\ treated}$	*95% Confidence limits*
Noradrenaline	Dipyridamole	4	1.2	(0.9–1.6)
	Hexobendine	4	0.8	(0.5–1.2)
	Dilazep	4	1.2	(0.8–1.8)
Aminophylline	Dipyridamole	5	0.8	(0.5–1.4)
	Hexobendine	4	0.7	(0.5–0.9)
	Dilazep	4	1.3	(0.7–2.6)
Prostaglandin E_2	Dipyridamole	12	1.3	(0.9–2.0)
	Hexobendine	8	1.3	(0.8–2.3)
	Dilazep	5	1.0	(0.7–1.6)
Glyceryl trinitrate	Dipyridamole	7	2.4	(1.3–4.5)
	Hexobendine	7	0.7	(0.5–1.0)
	Dilazep	5	1.0	(0.7–1.5)

that of adenosine and responses to these two purines were potentiated to a similar extent by drugs which block adenosine uptake. The latter effect is somewhat surprising since ATP, unlike adenosine, does not readily cross cell membranes (Lowy, Davoll & Brown, 1952; Hoffman & Okita, 1965; Hattori, Miyazaki & Nakamura, 1969). A possible explanation is that the spasmolytic effect of ATP depends on its first being hydrolysed to adenosine at the cell surface, a reaction which is known to occur rapidly in the heart (Baer & Drummond, 1968) and in the gut (Burnstock, 1972) and may, therefore, also occur in the trachea.

In contrast, the spasmolytic potency of adenine was virtually unaltered by drugs that block adenosine uptake. This suggests that adenine is not actively transported into tracheal smooth muscle cells or that its uptake process is not sensitive to dipyridamole-like drugs. It may be significant that adenine uptake into guinea-pig heart muscle is not inhibited by dipyridamole (Kolassa *et al.*, 1970) and so in this tissue at least, adenine and adenosine enter the cell by different processes.

Since inosine uptake into guinea-pig heart muscle has been reported to be inhibited by dipyridamole (Kolassa *et al.*, 1970) it was expected that dipyridamole would enhance the spasmolytic potency of inosine in the guinea-pig trachea. In fact, its activity was greatly reduced by dipyridamole and by the other two adenosine uptake blocking drugs. The mechanism of the spasmolytic action of inosine may, therefore, be intracellular and quite different from that of adenosine. Dipyridamole and similar drugs would then antagonize the effects of inosine by preventing its uptake into cells. The same explanation could also apply to guanosine, which behaves like inosine. Tachyphylaxis to the relaxant effects of inosine and guanosine on the trachea develops rapidly and may, therefore, depend on their releasing some endogenous spasmolytic substance from a depletable intracellular store, but the nature and origin of such a substance are not known.

The specificity of action of the adenosine uptake blocking drugs on the trachea is clear, since they greatly enhance the spasmolytic effect of adenosine and ATP but have little or no effect upon that of adenine, noradrenaline, aminophylline, prostaglandin E_2 or glyceryl trinitrate. However, the small enhancement of the response to glyceryl trinitrate seen with dipyridamole (1 μg/ml) is of interest as dipyridamole has also been reported to enhance responses to glyceryl trinitrate on guinea-pig parametrial artery by an unexplained mechanism (Bell, 1974).

A considerable amount of evidence exists for the presence, in gastro-intestinal smooth muscle, of intramural inhibitory nerves which are neither adrenergic nor cholinergic (Burnstock, 1972) and Burnstock and his co-workers have proposed that adenosine or an adenine nucleotide is the neurotransmitter involved. The presence of non-adrenergic, non-cholinergic inhibitory nerves in the guinea-pig trachea has also been reported (Coburn & Tomita, 1973; Coleman & Levy, 1974) and there is some evidence that the neurotransmitter may be an adenyl derivative (Coleman & Levy, 1974). A possible criticism of the idea of purinergic nerves in the trachea is that exogenously applied adenosine derivatives appear to be less active than known nerve transmitters. However, the present findings reveal that the 'true' potency of adenosine and related nucleotides may be considerably greater than originally thought. The order of potency of the purine derivatives in the untreated trachea—adenine $\geqslant$ guanosine $>$ inosine $\geqslant$ adenosine $\geqslant$ ATP—becomes adenosine $\geqslant$ ATP $\gg$ adenine $\gg$ guanosine $=$ inosine after blockade of the adenosine uptake process. In the presence of dipyridamole, the potency of adenosine approaches that of the sympathetic neurotransmitter noradrenaline. Adenosine and adenine nucleotides need no longer be precluded as possible neurotransmitters on the grounds of low potency.

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A COMPARISON OF THE TIME COURSE OF EXCITATION AND INHIBITION BY IONTOPHORETIC DECAMETHONIUM IN FROG ENDPLATE

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1 The depolarization of frog endplate by a brief iontophoretic application of decamethonium was slower in time course than the inhibition of a long carbachol response produced by the same decamethonium pulse, or than the excitation produced by a brief equipotent carbachol pulse.

2 The delay between the peak inhibition and peak excitation produced by decamethonium, about 50 ms, is too great to be explained by slow receptor activation kinetics, since Katz & Miledi (1973) have shown that the lifetime of decamethonium-activated receptors is only 0.25 milliseconds.

3 If doses of carbachol and decamethonium are adjusted to give response amplitudes in the ratio corresponding to the ratio of their presumed maximum responses, then there is little difference in the time courses of the responses.

4 This observation, together with the finding that increasing the dose applied slows the decamethonium response much more than the carbachol response, suggests that a decamethonium response contains contributions from a much wider area of receptive membrane than does a carbachol response of equal amplitude.

5 Simulation shows that these geometrical effects are sufficient to account for the rapidity of inhibition compared to excitation without postulating slow receptor kinetics.

6 It is pointed out that similar effects may account for certain results obtained in iontophoretic studies of desensitization.

Introduction

In 1957 del Castillo & Katz (1957a, b) suggested that agonists activate receptors in a two-stage process: $A + R \rightleftharpoons AR \rightleftharpoons AR^*$, where AR is an inactive intermediate complex, which then isomerises to give the active complex AR*. Their main evidence for this was that certain agonists would inhibit the action of others which they took to indicate that the agonist could combine with the receptor without activating it. Perhaps the strongest evidence for a sequential mechanism was that depolarizations produced by iontophoretic decamethonium pulses were much slower than those produced by carbachol, and that this difference seemed too great to be explained by diffusion alone. Furthermore, an effective inhibition of acetylcholine existed very early in the decamethonium response, suggesting that the decamethonium had combined with receptors, but only subsequently activated them.

The present work was undertaken initially to check, extend and quantitate these effects. However it then became clear that although the phenomenon certainly existed, its explanation was to be sought in terms of diffusional delays and the geometrical complexities of the iontophoretic method, rather than a kinetic receptor model. An abstract describing this work has already appeared (Adams, 1974).

Methods

Recordings from superficial endplates of sartorius muscles of *Rana temporaria* or *R. pipiens* were made with conventional intracellular techniques. The experiments were performed at room temperature (18–22°C) using a Ringer of the following composition (mM): Na 113.5, K 1.9, Cl 114.1, HCO_3 2.4, H_2PO_4 0.064 and Ca 1.2. Two or three barrelled pipettes for iontophoresis contained decamethonium iodide (Koch-Light), carbachol chloride (Koch-Light) and 0.9% w/v NaCl solution (saline). The retaining currents on both drug barrels were initially set to high values. When a sensitive endplate region was located, the carbachol retaining current was then reduced until small pulses gave carbachol responses which showed no signs of desensitization. Finally the decamethonium barrel retaining current was reduced almost to the

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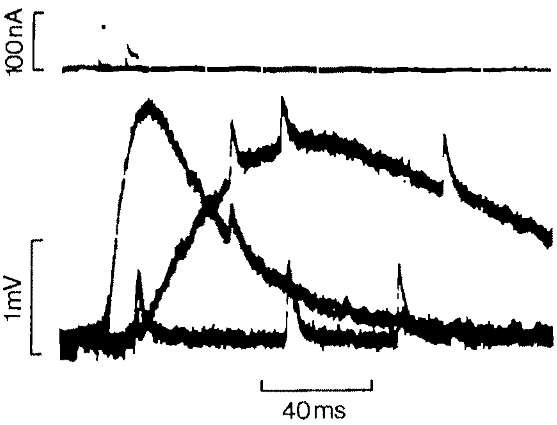

Figure 1 Comparison of time courses of depolarization by brief carbachol or decamethonium pulses. Upper beam: pipette current. Lower beam: membrane potential. Three records were superimposed: a response to carbachol (fast depolarization), a response to decamethonium (slow depolarization) and a base line. Miniature endplate potentials are present on all records. Carbachol and decamethonium were applied from separate barrels of a twin pipette.

point where a modification of the carbachol response became detectable. In order to obtain responses to long (0.5–1 s) pulses of carbachol which superimposed exactly on successive trials (see Figures 2 and 4 and also Adams, 1974) the repetition rate had to be very slow (<0.03 Hz). The arrangements for voltage-clamp have already been described (Adams, 1975a). The iontophoretic currents were monitored in all experiments although they are not always shown in the records.

Results

Inhibition precedes excitation

Figure 1 shows an experiment which simply reproduces the original observation of del Castillo & Katz (1957a), that depolarizations produced by a short pulse of decamethonium from one barrel of a pipette are considerably slower than depolarizations produced by a pulse of carbachol from another barrel. In these experiments the pipette could always be manipulated to give carbachol responses with times to peak <20 milliseconds. However in these positions decamethonium responses of similar amplitude had times to peak of ~80 milliseconds. It was also noted, in agreement with del Castillo & Katz, that even very large decamethonium pulses would only give small (<10 mV) depolarizations, while there seemed to be no such limit on the size of carbachol responses. This agrees with the quantitative observations on the dose-response curves (Adams, 1975b).

Attempts were made to measure the inhibition existing at various times in the decamethonium response by eliciting carbachol responses at various times. However this technique proved unsatisfactory since it requires carbachol responses which are very short compared to the rise-time of the decamethonium response, and though the carbachol responses alone met this requirement, in the presence of decamethonium they became very rounded.

Therefore the following method was adopted. Having positioned the pipette tip close to the receptors (as judged by carbachol responses with <20 ms rise-times), a short pulse was then applied to the decamethonium barrel and the resulting slow depolarization recorded. A long (500 ms–1 s) pulse was then applied to the carbachol barrel. Because of the nature of diffusion from a point source (Waud, 1967) the resulting depolarization rose very slowly, and did not always reach a plateau before the end of the expelling pulse. However it was judged that even longer pulses increased the likelihood of desensitization, which would affect the closest receptors most, slow up the responses, and mask the effect being sought. Finally, the long carbachol pulse was reapplied, and the decamethonium pulse also applied toward the end of the carbachol response. Provided that the response to decamethonium alone was rapid, a rapid inhibition of carbachol depolarization occurred, which sometimes passed over into an excitation. This method, and the results obtained, are shown in Figure 2 (see also Adams, 1974).

'Inhibition' and 'excitation' are taken to refer to responses to decamethonium plus carbachol relative to the original carbachol trace. It was usually quite clear that the decamethonium inhibition peaked earlier than the original decamethonium depolarization. In 12 experiments the time to peak of the control decamethonium depolarization was 88 ± 9.6 ms (mean ± s.e.) and the time to peak of the inhibition was 38 ± 4.5 ms (the times being measured in both cases from the start of the decamethonium pulse). The ratio of the times to peak for excitation and inhibition was 2.4 ± 0.3.

Is the delay artefactual?

The simplest artefact liable to occur in this type of experiment is interbarrel coupling, leading to iontophoresis of one compound when another was intended. Although a spacer was not used, two observations indicated that this effect was not important in the present experiments. First, in some experiments a triple barrelled pipette was used, one barrel being filled with saline. Pulses to this barrel did not expel carbachol or decamethonium from the other barrels. Second, the depolarization produced by a

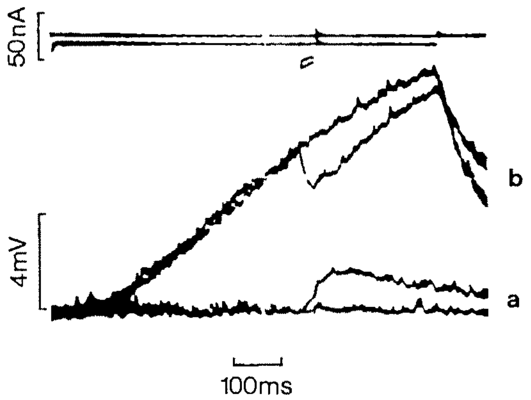

Figure 2 Comparison of time courses of inhibition and excitation by iontophoretic decamethonium. The traces marked (a) show a base line and a depolarization produced by a decamethonium pulse (current monitored in top beam). The traces marked (b) show 2 successive depolarizations produced by a long carbachol application, during one of which the decamethonium pulse was also applied. Note that the carbachol+decamethonium trace falls more slowly than the carbachol alone trace at the end of the pulse.

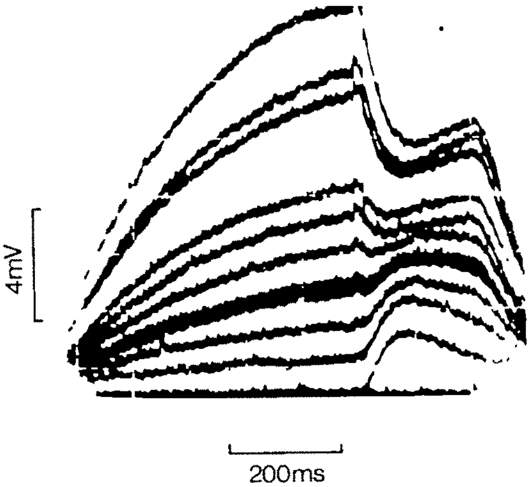

Figure 3 Decamethonium responses during varying carbachol backgrounds. The records show a base line, a response to decamethonium alone, and then decamethonium responses to the same dose during increasing carbachol backgrounds. Note the short upward deflections preceding the decamethonium inhibitions. The base line appears half wave rectified because of occlusion by the oscilloscope mask.

pulse to the decamethonium barrel was reduced or eliminated by increasing the backing voltage applied to the decamethonium barrel, but was unaltered by increasing the backing voltage applied to the carbachol barrel, or *vice versa*. Thus it seemed unlikely that the effects ascribed to one drug were due to the other.

A second possible problem was mentioned above, that the long carbachol application might desensitize the receptors immediately under the pipette tip. However this effect would tend to slow the decamethonium responses (whether excitatory or inhibitory) and could not explain the above results. Some evidence that this process could occur was obtained when comparing the decamethonium inhibitions at different strengths of carbachol application (Figure 3), since the inhibitions were slowed by increasing the carbachol background. Another possible explanation for this is considered below.

A third artefact was the occasional unexpected appearance of triphasic decamethonium responses during background carbachol. This effect is visible in Figure 3, where it will be noted that during intense carbachol action the decamethonium inhibition is preceded by a depolarization. On close examination it became apparent that this early 'excitation' coincided with the decamethonium expelling pulse itself and had the form of a very short electrotonic potential. However, it was quite clear that the electrode was not 'semi-intracellular' (del Castillo & Katz, 1955) since even a large pulse to the decamethonium barrel alone produced no such effect. This initial depolarizing response showed the following additional features. Its amplitude was proportional to the intensity of carbachol action. It was still present when the decamethonium barrel backing voltage was increased sufficiently to prevent completely subsequent decamethonium responses. It could also be seen when a pulse to the saline barrel was applied during intense carbachol action. It was concluded the local carbachol action lowered the resistance of the membrane under the pipette tip to allow access of the decamethonium expelling current to the interior of the fibre, producing a reversible 'pharmacological' impalement.

A fourth artefact was anticipated that might theoretically account for the main result noted above. This was that the membrane resistance might be sufficiently lowered by carbachol action to reduce significantly the fibre time constant. This would tend to speed up responses to concurrently applied decamethonium (whether excitatory or inhibitory). However in voltage clamp experiments the inhibition still clearly preceded excitation (Figure 4). In 2 experiments the mean times to peak of inhibition and excitation were 12 and 26 ms respectively, so although this factor might operate to some extent in the potential recording experiments, at least part of the delay remains to be accounted for.

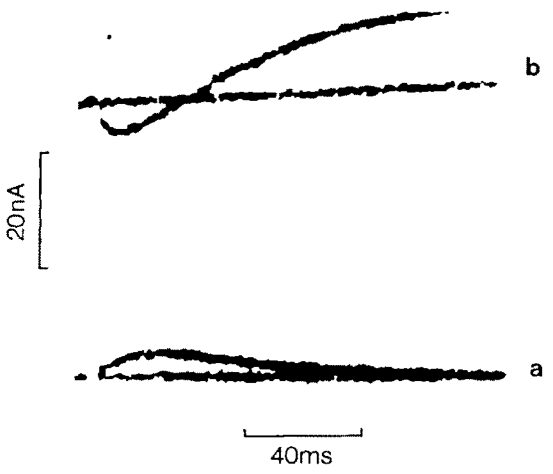

Figure 4 Decamethonium inhibition and excitation under voltage clamp. The clamp current was recorded separately from the iontophoretic current across a resistor in the feedback circuit. Decamethonium alone (a) produced a monophasic response, whereas superimposed on carbachol (b) it is diphasic. (For further explanations see Figure 2).

Considerations mitigating against a 'kinetic' interpretation

One can idealise the present experiments in terms of the 2 step model of receptor activation outlined in the Introduction. Suppose that a very short pulse of decamethonium is applied to the receptors, which then binds very rapidly, so that immediately after the pulse the intermediate AR concentration is raised instantaneously to a finite value, while both [AR*] and free [A] are negligible. This situation can be represented

$$A + R \xleftarrow{k} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^*$$

(This situation will not occur during the pulse, but it can be taken to obtain from the peak of the inhibition, which is taken as $t = 0$).

An expression for [AR*] as a function of t is obtained by solving the equations in d[AR]/dt and d[AR*]/dt. The time at which [AR*] reaches a peak, t_{max}, is obtained by setting the derivative of this expression equal to zero and solving for t. One obtains $t_{max} = 1/(r_1 - r_2) \ln (r_2/r_1)$, where r_1 and r_2 refer to the positive and negative roots respectively of the equation

$$r_1, r_2 = [-(\beta + k + \alpha) \pm \sqrt{(\beta + k + \alpha)^2 - 4k\alpha}]/2.$$

Since [AR] is maximal at $t = 0$ and [AR*] at $t = t_{max}$, t_{max} gives the delay between peak inhibition and excitation. Since the maximum decamethonium response is small $\beta \lll \alpha$. Also, Katz & Miledi (1973) have shown that for decamethonium $\alpha \sim 0.25$ ms^{-1}. Clearly t_{max} will not at all reflect the numerical value of $1/\beta$.

These considerations suggest that an alternative explanation of the observed delay should be sought. The simplest assumption would be that true equilibrium exists throughout the responses, and that the form of the responses is determined by access factors.

An equilibrium interpretation

Since the efficacy of decamethonium is much less than that of carbachol, to obtain decamethonium and carbachol responses of equal amplitude many more receptors must combine with decamethonium than need to combine with carbachol. The main way in which additional receptors are recruited by increasing the 'dose' is probably not by increasing the local concentration of decamethonium around a fixed area, but by spreading the area of receptors activated. If the amplitude of responses to decamethonium and carbachol are adjusted to be in the ratio of their extrapolated maximum responses in bath application experiments (Adams, 1975b), then the responses differ very little in their time courses (Figure 5a). Any residual difference can probably be explained by a slower diffusion of decamethonium. Whereas increasing doses of carbachol evoke responses of approximately similar time-course, increasing doses of decamethonium produce progressively slower responses, as though successively wider areas of receptors were being activated (Figures 5 & 6).

So one can suggest that in the interaction experiments described above the decamethonium excitation would be slow, because the receptors involved are distributed over a wide area, whereas the inhibition of carbachol is fast, since carbachol action is confined to the receptors immediately under the pipette tip, and decamethonium can only inhibit carbachol where carbachol is present.

To test this idea a simple computer simulation of the postulated situation was set up. The receptors were considered as an infinite uniform plane located a vertical distance λ under the pipette tip. The receptor plane was divided into a series of concentric annuli, the nth annulus having a radius of n microns. Decamethonium and carbachol concentrations, normalized with respect to their respective receptor binding constants, were assumed for the central annulus (D_0 and A_0). The average decamethonium concentration at the nth annulus was calculated assuming that the expelling pulse was very short compared to the responses. The appropriate diffusion equation (del Castillo & Katz, 1955) is then

$$D_r = \frac{Q}{8(\pi k t)^{1.5}} \exp(-r^2/4kt)$$

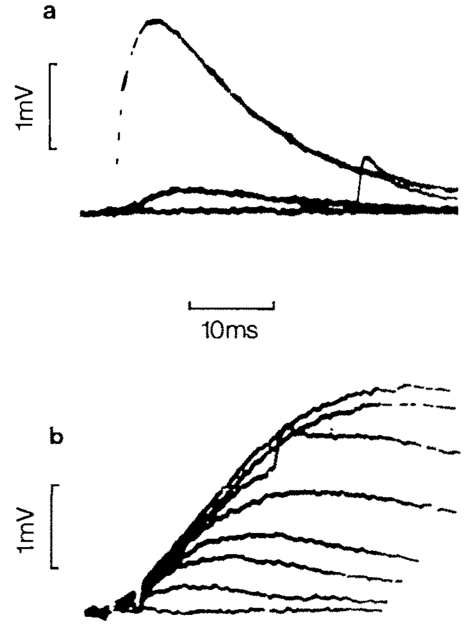

Figure 5 (a) Comparison of time courses of depolarizations produced by carbachol or decamethonium whose amplitudes were in the ratio 7 to 1. One carbachol and two decamethonium responses (during one of which a miniature endplate potential occurred) superimposed on a base line. (b) Effects of increasing decamethonium dose at the junctional spot shown in (a).

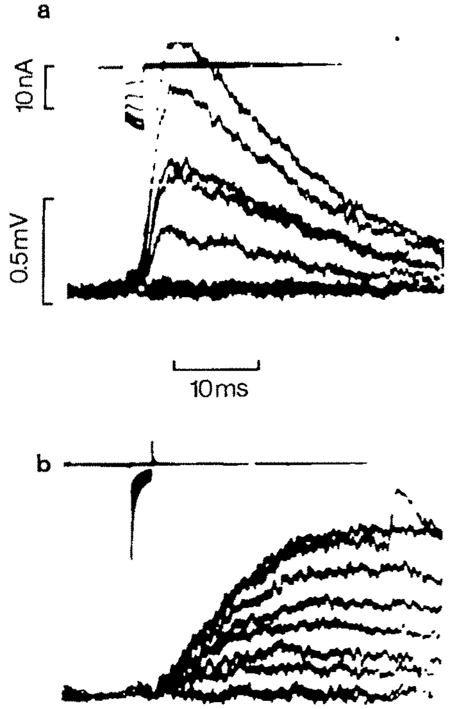

Figure 6 Comparison of effect of increasing carbachol (a) or decamethonium (b) doses. Note that the rise time of the miniature endplate potential occurring in (b) is only slightly faster than the smallest carbachol response shown in (a).

where k is the diffusion coefficient of decamethonium, Q is the quantity expelled, D_r is the concentration at a distance r from the point source, and t is time. This can be reworked to give the concentration at the nth annulus in terms of the peak concentration at the *zero*th annulus, $D_{o,max}$

$$D_n = \frac{\lambda^3 \exp(-X_n^2/4kt) \exp 1.5}{(6kt)^{1.5}} D_{o,max}$$

where X_n is the average distance of the nth annulus from the pipette and λ is the vertical distance of the source from the receptive plane. An approximate expression for the carbachol concentration at the nth annulus, A_n, was obtained assuming that the expelling pulse was sufficiently long for a steady state to be achieved. Then

$$A_n = \left(\frac{\lambda}{X_n}\right)^2 A_o$$

This simplifies the calculation although even for very long pulses a true steady state may not be achieved (Waud, 1967). Y_n, the fraction of the receptors at the nth annulus activated by the combination $A_n + D_n$, was obtained assuming

$$Y_n = \left[\frac{A_n + D_n/\sqrt{Z}}{A_n + D_n + 1}\right]^2$$

where Z is the ratio of the maximum responses to carbachol and decamethonium (Adams, 1975c). The relative response of the nth annulus, R_n, was then calculated using the relation $R_n = \pi(2n-1)\,(Y_n)$.

The programme caused n to be incremented from zero, and corresponding values of R_n to be calculated and summed to give a total response R_s, until adding a further value of R_n caused R_s to change by less than some criterion (usually 0.01 or 0.05), when the calculation terminated and the final value of R_s was presented. This process was then repeated for other suitable values of t. Figure 7 shows a typical set of calculations for a fixed value of $D_{o,max}$ and increasing values of A_o (corresponding to the experiment shown in Figure 3). The model predicts quite successfully the slow excitation, the initial early inhibitions, and the rightward shift of the inhibition peak with increasing carbachol applications. The main defect is that the tails of the decamethonium responses tend to be rather

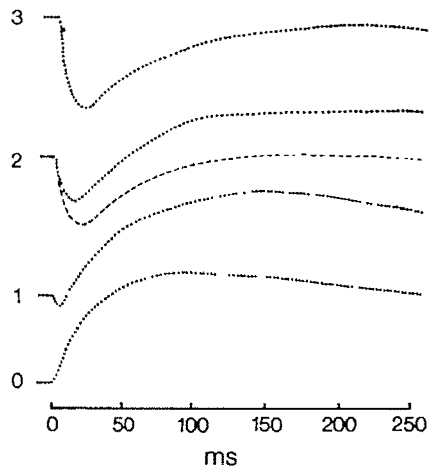

Figure 7 Simulation of interaction of iontophoretic carbachol and decamethonium. A double barrelled pipette a vertical distance λ above the receptive plane delivers a short pulse of decamethonium and a long pulse of carbachol. The total endplate response is computed as the sum of the responses of concentric annuli of inner radius n µm. The curves show responses to a fixed dose of decamethonium in the presence of increasing background doses of carbachol. The ordinate is the relative response (arbitrary units). The following parameter values were inserted in the text equations. $D_{0,\max}=2$; $A_0 = 0, 1, 2, 3$; $\lambda = 5$ µm; $k=0.6$ µm^2 ms^{-1}; $Z=10$; criterion$=0.05$. The dashed curve shows the effect of slightly increasing Z (to 13.5). See text for further explanations.

prolonged, but this is probably merely due to the (in retrospect) rather poor assumption of infinite plane geometry.

Discussion

It seems quite clear that the experimental observations can be explained without postulating special kinetic behaviour merely by differences in the spatial distribution of carbachol and decamethonium, arising mainly from the greater relative efficacy of carbachol. In principle it might be possible to repeat the experiments using bath application of carbachol to avoid the problem of spatial inhomogeneity, but probably any delay due to slow receptor activation would be undetectably small in any case. An alternative would be to use a very small isolated patch of receptors, perhaps by blocking off all the surrounding receptors by bungarotoxin treatment. It certainly seems plausible that some of the rate constants for conformational change of the receptor are slow compared to the potential rapidity of iontophoresis. But unfortunately most experiments will tend to yield overall rate constants in which the contribution of 'slow' steps is masked by that of 'fast' steps.

Considerable caution must be exercised in placing kinetic interpretations on the results of iontophoretic experiments (see for example, Steinbach, 1971). As a further example of the possible distortions that complex geometry may introduce, it is interesting to consider the desensitization experiments of Katz & Thesleff (1957), which share with the present experiments the feature of superimposing short test pulses on long conditioning pulses. Katz & Thesleff found, rather unexpectedly, that the rate of desensitization by acetylcholine or carbachol first decreased with increasing agonist concentration and then reached a minimum and started to increase. In bath application experiments only increasing desensitization rate with increasing agonist concentrations is seen (Rang & Ritter, 1970; Adams, 1975a). In the iontophoretic experiments the desensitization rate was estimated as the rate constant for decline in amplitude of repetitive agonist responses superimposed on a prolonged conditioning application. The test agonist pulses will produce responses which contain components from receptors located at various distances, the more distant receptors contributing slower, but in the absence of a conditioning dose, smaller responses. The conditioning dose produces 2 effects: (a) desensitization of receptors, the more distant receptors desensitizing more slowly; (b) modification of the responses of undesensitized receptors to the test dose. This second effect, arising from the sigmoidicity of the dose-response curve (Katz & Thesleff, 1957; Adams, 1975b) consists qualitatively of a reduction of the contribution of near receptors (because these receptors are activated by agonist concentrations which are near the top of the dose-response curve) and an increase in the contribution of distant receptors (since here the agonist concentration is near the bottom of the dose-response curve). The validity of this analysis is confirmed by the finding that the response to a test dose is slowed by the conditioning dose and that this effect, unlike the desensitization, appears immediately on applying the conditioning dose (Katz & Thesleff, 1957; Adams, unpublished observations). Combining (a) and (b), one can see that while increasing the conditioning dose will increase the rate of desensitization at any fixed site, there will be an additional tendency for the apparent overall rate to decrease, because increasing the conditioning dose increases the relative importance of contributions to the test response from more distant receptors which are desensitizing at slower rates. It is difficult to predict which of these effects might predominate for any given range of conditioning doses. However this discussion shows that the decrease in the desensitization rate observed by Katz & Thesleff might be due to the geometrical complexities of the iontophoretic situation rather than to unusual features of desensitization kinetics. Other differences between iontophoresis and bath application desensitization findings, for example the different relationships between the rate of desensitization and the final desensitization achieved (compare Katz & Thesleff, 1957, Figure 2 and Adams, 1975a, Figure 4) can be explained in the same way.

Note added in proof. The simulations shown in Figure 7 have been repeated assuming a finite line receptor distribution and a linear decrease of agonist concentration with distance in the steady state. Under these conditions qualitatively identical results were obtained.

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CONCENTRATION OF (±)-PROPRANOLOL IN ISOLATED, PERFUSED LUNGS OF RAT

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1 The metabolism and the accumulation of (±)-propranolol have been studied in isolated lungs of the rat, perfused with an artificial medium.

2 Little or no metabolism took place during the perfusion periods (up to 10 minutes).

3 Accumulation was observed with high tissue/medium ratios for substrate concentrations of 0.2 μM to 1 mM; there was evidence for saturability, but no real plateau could be seen. The presence of two binding sites with different affinities was established.

4 Cold greatly inhibited the accumulation process at low substrate concentrations, but had no effect at 1 mM propranolol.

5 Inhibition of accumulation was measured in the presence of imipramine, desmethylimipramine, nortryptiline, chlorpromazine and of Na^+-free medium. Cocaine, 5-hydroxytryptamine and noradrenaline had no effect. Lidocaine enhanced the accumulation process. Release of previously bound propranolol was accelerated in the presence of propranolol and imipramine, unaffected by a Na^+-free medium and decreased by cold and by lidocaine.

6 Experiments on lung tissue slices yielded qualitatively similar results to those obtained with perfused lungs. Ouabain and KCN had no or little effect on propranolol accumulation.

Introduction

Previous studies on the distribution of (±)-propranolol in tissues have shown that the lungs were able to concentrate (±)-propranolol to a remarkable extent since a tissue/medium ratio of up to 250 was observed (Black, Duncan & Shanks, 1965). Further analysis (Hayes & Cooper, 1971) showed that in rats, dogs and monkeys, large amounts of metabolites of propranolol could also be detected in the lungs. It was not known if the presence of these metabolites was the result of the local pulmonary metabolism of propranolol or of their accumulation after production elsewhere in the body.

The present studies were therefore undertaken to investigate the mechanism of concentration and the possible metabolism of (±)-propranolol in isolated perfused lungs of the rat. In view of their similar physicochemical properties, it was also interesting to compare the mode of accumulation of propranolol with that of imipramine and other basic amines, whose concentration in the lungs has already been the object of recent studies (Junod, 1972a, Andersen, Orton, Pickett & Eling, 1974). The possibility of a type of uptake similar to the amine pump had to be examined in view of the effect of propranolol on noradrenaline uptake by the heart as reported by Foo, Jowett & Strafford (1968), although, according to Iwasawa & Gillis (1974), propranolol had no effect on noradrenaline uptake by the lung.

Methods

Medium and materials

The perfusion medium consisted of Krebs-Ringer bicarbonate buffer, containing 5 mM glucose and 4.5% bovine serum albumin; to obtain a Na^+-free medium, sucrose was substituted for NaCl and Tris HCl for $NaHCO_3$, while the albumin was made Na^+-free according to the procedure already described (Junod, 1972b). [^{14}C]-propranolol hydrochloride (14.66 μCi/mg or 4.377 mCi/mmol), (±)-propranolol, 4-OH propranolol and desisopropylpropranolol were all given by ICI. Imipramine and desmethylimipramine were given by Geigy Pharmaceuticals, iproniazid phosphate by Roche Products Ltd., nortryptiline by E. Lilly & Co. Ltd., chlorpromazine hydrochloride by May and Baker Ltd. and lidocaine hydrochloride by Astra Chemicals Ltd. Ouabain, noradrenaline bitartrate and 5-hydroxytryptamine creatinine sulphate were purchased from BDH. Bovine serum albumin fraction V was obtained from Sigma Chemicals.

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Unless otherwise specified, drugs were added to the perfusion medium and were therefore present during the entire perfusion. In the experiments where the effects of drugs on the efflux of propranolol were studied, drugs were added to the perfusion medium only during the period when efflux of ^{14}C was measured.

Experimental procedures

Details of the procedure used have already been reported (Junod, 1972a). Male Wistar rats, weighing 200–300 g, were used. After anaesthesia with 50 mg/kg Na pentobarbitone injected intraperitoneally and tracheostomy, the animal was artificially ventilated with a gas mixture of 95% O_2 and 5% CO_2. The lungs were then isolated, perfused at the rate of 10 ml/min and placed in a closed chamber. After a 5 min equilibration period, the lungs were perfused with medium containing various concentrations of [^{14}C]-propranolol for various periods of time. They were then dissected, blotted dry, weighed and homogenized in 6 ml 0.4 N $HClO_4$. When the efflux of ^{14}C was measured, the effluent was collected during the 8 min following the perfusion with [^{14}C]-propranolol and its radioactive content was measured and expressed as % of the initial uptake calculated from the inflow-effluent difference during the 5 min perfusion with [^{14}C]-propranolol.

When rat lung slices were used, the lungs were perfused *in situ* with 0.9% w/v NaCl solution (saline) at 4°C to get rid of the blood and subsequently sliced. Slices were then incubated for 60 min in 2.5 ml standard medium (without albumin) containing 1 μM [^{14}C]-propranolol and another drug when necessary, at 37°C, in 5% CO_2 and 95% O_2. At the end of the incubation period, slices were blotted dry, weighed, dissolved in Soluene (Packard) and their radioactive content measured.

Analytical procedures

The radioactive content of samples of inflow, effluent and lung homogenate was measured in a scintillation spectrometer after addition of 10 ml of Instagel (Packard). An external standard was used to monitor counting efficiency and radioactivity measurements were corrected accordingly. The tissue/medium ratio was calculated from the ratio

$$\frac{\text{d min}^{-1}\ \text{g}^{-1}\ \text{lung tissue}}{\text{d min}^{-1}\ \text{ml}^{-1}\ \text{inflow}}$$

In the studies on [^{14}C]-propranolol metabolism by perfused rat lungs, the lungs were homogenized in 10 ml 10% Na_2CO_3 at pH 10. An aliquot of lung homogenate was extracted with 17 ml toluene. Another aliquot was acidified to pH 1–2 and extracted with ethyl acetate. The radioactivity in the solvent phase was measured and compared with that of the lung homogenate. The toluene extract was then concentrated under a stream of N_2 and an aliquot of the concentrate was analyzed by thin-layer chromatography on glass plates coated with Silica Gel F-254. The solvent system was : isopropanol: ammonia: water (20:1:3) and good separation was obtained between propranolol, 4-OH propranolol and desisopropylpropranolol after a 6 h development.

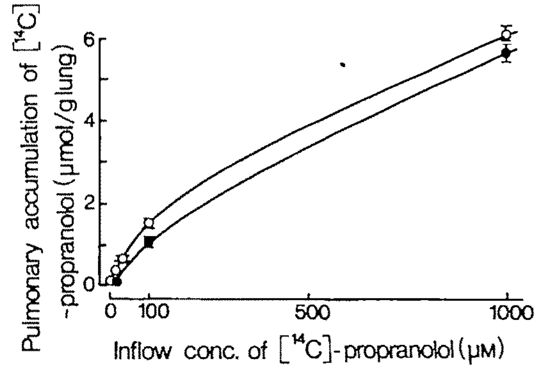

Figure 1 Relationship between the inflow concentration of [^{14}C]-(±)-propranolol and its accumulation in the lung after a 5 min perfusion period, at 37°C (○) and at 4°C (●). Each point represents the mean of 3 to 11 experiments. Vertical lines show s.e. mean.

Results

The percentage of ^{14}C extracted in toluene at pH 10 was 81.2 ± 2.6% (mean of 13 determinations ± s.e.) for propranolol concentrations in perfusion medium ranging from 1 to 34 μM. After a second extraction of the aqueous residue, 9.3 ± 1.2% of the ^{14}C initially present in the lung homogenate was present in the solvent phase. Ninety per cent of the radioactivity was therefore extractable in toluene at alkaline pH, and chromatographic analysis of the toluene extracts established that all of that radioactivity could be attributed to propranolol. Extraction of lung homogenate in ethyl acetate at pH 1–2 resulted in the presence of 10.6 ± 1.3% of the ^{14}C present in the lung homogenate in the solvent phase. No chromatographic analysis of that fraction was made. The radioactive content of the lung was taken as a measure of the [^{14}C]-propranolol accumulation in the lung, since, to the extent of at least 90%, it corresponded to [^{14}C]-propranolol, and no correction factor was applied.

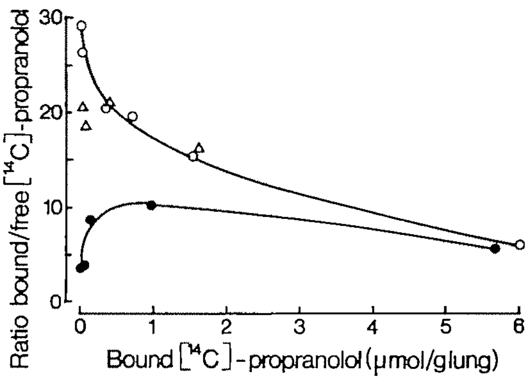

Figure 2 Relationship between the [^{14}C]-(±)-propranolol bound to the lung and the ratio of bound/free (^{14}C]-(±)-propranolol during a 5 min perfusion period, at 37°C (○), 27°C (△) and 4°C (●). Each point represents the mean of 3 to 11 experiments.

The effect of the duration of perfusion with 1 μM propranolol on its pulmonary accumulation was slight, since the tissue/medium ratio per unit of time decreased from 6.1 ± 0.2 to 5.2 ± 0.5 and 4.6 ± 0.6 for periods of 2, 5 and 10 min (mean ± s.e. of four, eleven and four experiments, respectively). In the subsequent experiments 5 min perfusions with [^{14}C]-propranolol were used.

Increasing the substrate concentration from 0.2 μM to 1 mM resulted in a progressive decrease in the tissue/medium ratio but no real plateau could be obtained, when substrate concentration was related to the accumulation of propranolol in the lung (Figure 1).

The effect of temperatures from 4°C to 37°C on the pulmonary concentration of [^{14}C]-propranolol at three different concentrations of substrate was measured. The inhibitory effect of low temperature was found to be dependent on the substrate concentration, being very marked at 0.2 and 1 μM [^{14}C]-propranolol and nearly absent at higher concentrations (100 and 1000 μM).

From these data, a Scatchard plot relating the amount of bound propranolol to the ratio of bound/free propranolol was drawn (Figure 2); it was certainly made of two different curves, establishing the existence of two populations of binding sites with different affinities. Only the high affinity binding sites were apparently sensitive to the inhibitory effect of low temperature.

Replacement of Na^+ in the perfusion medium by sucrose or Li^+ resulted in a decrease in the accumulation of [^{14}C]-propranolol, that effect being particularly marked in the presence of sucrose (Table 1). The same table shows the inhibitory effect of high concentrations of drugs also known as lipophilic basic amines (imipramine, desmethylimipramine, chlorpromazine, and nortryptiline): its magnitude was in fact similar to that resulting from the effect of propranolol itself. Noradrenaline, 5-hydroxytryptamine, cocaine and 4-OH propranolol, on the other hand, did not affect the pulmonary accumulation of propranolol. The significant increase in tissue/medium ratio in presence of 0.1 mM lidocaine was unexpected. The experiments on the efflux of [^{14}C]-propranolol (Table 2) showed that propranolol itself and

Table 1 Effect of various experimental conditions on the accumulation of [^{14}C]-propranolol by isolated lungs of rat

Experimental condition	n	*T/M ± s.e.*
Control	11	26.0 ± 1.1
Control 4°C	7	3.4 ± 0.3*
Na ± free medium (Na^+ replaced by Li^+)	4	19.1 ± 1.4*
Na ± free medium (Na^+ replaced by sucrose)	4	13.7 ± 0.6*
Noradrenaline 0.1 mM	4	27.4 ± 1.2
5-hydroxytryptamine 0.05 mM	3	28.4 ± 2.8
4-OH propranolol 0.1 mM	4	23.1 ± 1.5*
Cocaine 0.1 mM	4	29.7 ± 2.0
Imipramine 0.01 mM	4	23.9 ± 2.8
Imipramine 0.1 mM	4	15.4 ± 0.9*
Desmethylimipramine 0.1 mM	4	14.7 ± 0.9*
Nortryptiline 0.1 mM	4	12.7 ± 1.1*
Chlorpromazine 0.1 mM	4	13.6 ± 0.8*
Lidocaine 0.1 mM	6	36.1 ± 1.2*

The accumulation of [^{14}C]-propranolol during a 5 min perfusion period with 1 μM [^{14}C]-propranolol was measured under various conditions and expressed as d min^{-1} g^{-1} lung/d min^{-1} ml^{-1} inflow (T/M).
*Statistically different from control value ($P < 0.05$).

Table 2 Effect of various drugs and medium compositions on the efflux of [^{14}C]-propranolol from isolated lungs of rat

Experimental condition	n	*Loss of ^{14}C in 8 min as % of ^{14}C uptake*
Standard medium	4	34.25 ± 2.54
+ Propranolol 10 μM	3	61.52 ± 1.32*
+ Imipramine 10 μM	3	52.74 ± 4.90*
+ Lidocaine 0.1 mM	3	24.75 ± 2.60*
Li Cl substituted for Na Cl	3	29.95 ± 2.91
Standard medium at 4°C	3	15.38 ± 2.12*

After a 5 min perfusion period with 1 μM propranolol and standard medium, the efflux of [^{14}C]-propranolol was measured during a 8 min perfusion period under various conditions (mean ± s.e.). The uptake of [^{14}C]-propranolol was taken as the inflow-outflow ^{14}C difference measured during the initial 5 min perfusion period.
*Statistically different from control values ($P < 0.05$).

imipramine had an accelerative effect; Na+-free medium did not result in any significant change, whereas lidocaine decreased that efflux. A more important reduction of efflux resulted from perfusion at low temperature.

Finally, experiments were done with lung slices to study the effects of metabolic inhibitors and also to see if qualitative changes in the nature of the process of concentration could be observed under these experimental conditions (Table 3). Under control conditions, the tissue/medium ratio obtained was 7.68. This value was sufficiently high to make the correction for the radioactivity present in the extracellular space unnecessary. Lowering the temperature and adding unlabelled propranolol and chlorpromazine had the same effect as in the perfused lungs. Ouabain, at 1 mM, did not decrease the tissue/medium ratio, whereas KCN, also at 1 mM, had only a partial inhibitory effect.

Discussion

The analysis of the radioactivity found in lung homogenate clearly indicates that little or no metabolism of propranolol took place under these experimental conditions. In view of the results obtained by Hayes & Cooper (1971), it is therefore logical to assume that the lung can accumulate large amounts of metabolites produced elsewhere in the body, in the liver in particular. On the other hand, Walle, Ishizaki & Gaffney (1972) reported that, during perfusion of isolated lung of the dog, isopropylamine could be formed from propranolol, but no quantitative data were given.

The nature of the mechanism of concentration of propranolol presents several points of similarity with that of imipramine (Junod, 1972a): it shows the same trend towards saturation at the same range of substrate concentrations; it is affected by the same basic, lipophilic drugs, but not by noradrenaline or by 5-hydroxytryptamine. The efflux of ^{14}C is accelerated by propranolol itself as well as by compounds of similar physicochemical properties.

Table 3 Effect of various drugs and conditions on the accumulation of [^{14}C]-propranolol in lung slices of rat

Experimental condition	n	*% T/M of control condition*
Standard medium	8	100
Standard medium at 4°C	4	48.7 ± 4.1
+ Propranolol 0.1 mM	4	34.4 ± 1.0
+ Chlorpromazine 0.1 mM	4	33.0 ± 2.0
+ Ouabain 1 mM	4	101.0 ± 3.7
+ KCN 1 mM	4	77.2 ± 4.8

Lung slices were incubated for 60 min in presence of 1 μM [^{14}C]-propranolol under various conditions. The tissue/medium ratio (T/M), measured as d min^{-1} g^{-1} lung/d min^{-1} ml^{-1} medium, is expressed as % of the tissue medium ratio obtained under standard conditions (mean ± s.e.).

However, the observations that both cold and Na+-free medium were inhibitory established a major difference. They strongly suggested the possibility of a Na+-dependent, carrier-mediated transport, as has been shown to be the case for the uptake of 5-hydroxytryptamine and noradrenaline by pulmonary endothelial cells (Junod, 1972b; Iwasawa, Gillis & Aghajanian, 1973; Nicholas, Strum, Angelo & Junod, 1974). In the case of propranolol, however, the effect of Na+-free medium was only partially inhibitory and varied depending on the nature of the substitute for Na+, Li+ or sucrose; the absence of effect of Na+-free medium on the efflux of propranolol is a strong argument against such a type of transport, since reversal of Na+ gradient should have resulted in an increased rate of release. The experiments done with lung slices in the presence of ouabain and KCN also support the view that propranolol accumulation does not depend on the existence of a normal Na+ pump. It is interesting to note that Gillepsie & Towart (1973) found a similar effect of sucrose and Li+ as substitutes for Na+ in their studies on the extraneuronal uptake of noradrenaline; they could not explain that observation satisfactorily.

Other studies (Huunan-Seppälä, 1972) have suggested the existence of a binding process to phospholipids, similar to that reported by Balzer, Makinose, Fiehn & Hasselbach (1968) for various lipophilic amines: chlorpromazine, prenylamine, reserpine. The same hypothesis was invoked in the case of imipramine concentration in the lung (Junod, 1972a), and the interrelations between drugs of similar nature are consistent with that concept.

That process appears to depend on the presence of sites with two different affinities, as can be seen from the Scatchard plot. A similar observation was reported by Huunan-Seppälä (1972) for binding of propranolol to mitochondria, by Kwant & Seeman (1969) for binding of chlorpromazine to red cells, by Balzer *et al.* (1968) for the binding of various amines to vesicles isolated from sarcoplasmic reticulum and by Weinstein, Varon & Roberts (1971) for binding of imipramine to brain synaptosomes.

Orton, Anderson, Pickett, Eling & Fouts (1973) studied the accumulation of several basic amines in isolated lungs of the rabbit, during steady state conditions. Their findings on the saturability of the concentration process correlate well with the results obtained with imipramine and propranolol in rat isolated lungs. Anderson *et al.* (1974) extended their studies on the nature of the concentration process of imipramine, amphetamine, chlorcyclizine and methadone. They were able to find evidence for the presence of two components of accumulation: a linear one and a saturable one, for high and low substrate concentrations respectively. The results obtained in this study are compatible with these findings (cf. Figure 1); it is suggested that the temperature-sensitive component and the linear component might be the expression of the same phenomenon, whose nature has yet to be defined. It is conceivable that, for the low affinity sites, hydrophobic bonds might exist, since the tissue/medium ratio does not exhibit temperature dependence.

All these results point to the existence of two different processes, defined by either their temperature-sensitivity or their kinetic characteristics. More work is needed before the exact pharmacological nature of these two processes and the location of these sites are known.

The direct implications of these concentration phenomena have already been discussed (Junod, 1972a, Anderson *et al.*, 1974), and are concerned with the relationship between binding and release of these drugs and their plasma concentration. It is clear that the lung can act as a capacitor, storing large amounts of these compounds and releasing them slowly. The magnitude of this role can be assessed if the degree of concentration of the drug in the lungs and its volume of distribution are known. The mode of administration has also to be considered, since the effect of pulmonary binding will be more apparent after intravenous injection than after oral ingestion. Except for methadone (Orton *et al.*, 1973), binding is not accompanied by metabolism. Since the binding is a reversible process, administration of another drug of similar physicochemical properties can result in the release of the previously stored agent and an unwanted increase in its plasma concentration. It is therefore necessary to study more closely the possible interrelationships between these products. The liver remains the most important organ for the long term disposal of these drugs, but the role of the lung in the short-term fate of drugs should not be neglected.

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INDIRECT EFFECTS OF AMINO-ACIDS ON SYMPATHETIC GANGLION CELLS MEDIATED THROUGH THE RELEASE OF γ-AMINOBUTYRIC ACID FROM GLIAL CELLS

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1 All experiments were performed on rat isolated desheathed superior cervical ganglia maintained in Krebs solution containing amino-oxyacetic acid (10 μM) at 25°C.

2 Influx rates of γ-amino-*n*-butyric acid (GABA) were measured by incubating ganglia in 0.5 μM [^{3}H]-GABA for 30 minutes. Influx was inhibited by 50% on adding 14.3 μM unlabelled GABA, 59.2 μM β-alanine (BALA) or 424 μM β-amino-*n*-butyric acid (BABA).

3 Efflux of [^{3}H]-GABA into non-radioactive solution superfused over ganglia previously incubated for 60 min in 1 μM [^{3}H]-GABA was measured. The mean resting efflux rate coefficient (k) was $0.64 \pm 0.05 \times 10^{-3}$ min^{-1}. Addition of high concentrations of unlabelled GABA, BABA or BALA to the superfusing solution increased k by (maximally) 3.6–4.3 times; half-maximal increases occurred at the following concentrations: GABA, 16 μM; BALA, 85 μM; BABA, 606 μM. Replacement of external Na$^+$ with Li$^+$ or TRIS increased the resting value of k and inhibited acceleration by external amino acids. Prior incubation in 1 μM [^{3}H]-GABA with 1 mM unlabelled GABA increased resting k 1.5 times, but did not alter the peak rate coefficient produced by external amino acids.

4 Neuronal depolarization produced by the amino acids was measured with surface electrodes. Pre-incubation in 1 mM GABA for 60 min potentiated low-amplitude responses to BALA or BABA but not those to GABA or 3-aminopropanesulphonic acid (a potent agonist with low affinity for the GABA carrier). Omission of external Na$^+$ reduced responses to BABA but increased those to GABA.

5 Incubation in 1 mM GABA for 60 min (as required to potentiate BABA or BALA actions) increased the amount of GABA in the tissue from 0.21 to 0.73 mmol/kg wet weight. Autoradiographs in which labelled GABA was used indicated that uptake into neuroglial cells was responsible for this accumulation.

6 It is suggested that: (i) BALA and BABA are substrates for the inward GABA carrier responsible for GABA entry into ganglionic glial cells; (ii) they accelerate efflux by inhibiting carrier-mediated re-accumulation of effluent GABA by the glial cells; (iii) interstitial GABA concentrations are thereby increased to a level capable of depolarizing adjacent neurones; and (iv) this, rather than direct GABA-receptor activation, accounts for the depolarization produced by low concentrations of BALA and BABA. Potentiation of their depolarizing action after pre-incubation in 1 mM GABA is suggested to result from the increased amount of intracellular GABA available for release, and is quantitatively compatible with this increase; inhibition in Na$^+$-free solution is due to their inability to inhibit re-accumulation of GABA under these conditions.

7 A model for the action of carrier substrates is described in an Appendix. Calculations based thereon yield increments in interstitial GABA concentration in the presence of carrier substrates compatible with those determined experimentally (up to 1 μM at rest or 3.4 μM after pre-incubation in GABA).

Introduction

There is no evidence to suggest that the central inhibitory neurotransmitter γ-aminobutyric acid

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(GABA) subserves any such function in sympathetic ganglia. Nevertheless, sympathetic ganglia share with brain two properties associated with GABA.

First, GABA increases chloride permeability in sympathetic neurones (Adams & Brown, 1975). This leads to a membrane depolarization (De Groat, 1970; Bowery & Brown, 1974). Although the direction of the potential change is opposed to that usually recorded in central neurones (Dreifuss, Kelly & Krnjević, 1969), the ionic permeability change is the same and available evidence suggests a close similarity between the receptors at the two sites with respect to ligand specificity.

Second, neuroglial cells surrounding sympathetic ganglion cells and fibres accumulate exogenous GABA through a high-affinity carrier-mediated transport system (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). This would appear to be broadly representative of a glial cell transport system elsewhere in the peripheral nervous system (Schon & Kelly, 1974) and in the brain (Henn & Hamberger, 1971; Schon, Beart, Chapman & Kelly, 1975; Iversen & Kelly, 1975).

The present experiments arose out of certain observations concerning the efflux of GABA from glial cells in sympathetic ganglia (Bowery & Brown, 1972; Bowery, 1974). When ganglia are pre-incubated with radioactively-labelled GABA and then washed with non-radioactive solution, the accumulated radioactivity leaves the ganglion at a slow steady rate. The rate of release is then increased on adding unlabelled GABA to the washing solution. A similar acceleration may be produced by other compounds which are transported into the glial cells via the GABA-carrier, such as β-alanine (BALA: Bowery & Brown, 1972; Bowery, Brown & Yamini, 1975, and unpublished observations; see also Schon & Kelly, 1975).

This raised two questions: (1) Could the substrate-accelerated release of GABA from the glial cells generate a sufficiently high interstitial concentration of GABA to depolarize the adjacent neurones? (2) Since there is some similarity (though not identity) between the structural requirements for a depolarizing agonist and a carrier substrate, to what extent might GABA-mimetic effects be generated indirectly through the release of GABA rather than through a direct effect on the neuronal receptors?

We have attempted to probe these questions using two compounds, β-alanine (BALA) and β-amino-*n*-butyric acid (BABA): both possess a higher affinity for the carrier than for the receptor, and so appeared prospectively suited for discerning such indirect GABA-mimetic effects. In this paper we describe first their interaction with the transport of labelled GABA in isolated sympathetic ganglia of the rat, and second some experiments on their depolarizing action on the ganglion. A model for the action of these compounds, and some calculations of both changes in GABA efflux rates and changes in interstitial GABA concentrations arising therefrom, are described in an Appendix. A brief note on the action of BABA has been published (Bowery, Brown & Marsh, 1975).

Methods

Superior cervical ganglia were isolated from rats (200–300 g, either sex) anaesthetized with urethane (1.5 g/kg). The connective tissue sheath was removed from the ganglion (and its attached pre- and postganglionic nerve trunks) and the preparation was maintained in Krebs solution at 25°C bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (mM) NaCl 118, KCl 4.8, $CaCl_2$ 2.52, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4$ 1.19, D-glucose 11 (pH 7.4). Na^+-free TRIS solution was prepared by replacing NaCl and $NaHCO_3$ with 143 mM TRIS-base (tris (hydroxymethyl) aminomethane) and adding 118 mequiv/l HCl to pH 7.4 (bubbled with 100% O_2).

Influx of GABA

The rate of inward GABA transport was measured from the amount of radioactivity accumulated after incubation in [^{3}H]-GABA. Isolated ganglia were incubated in Krebs solution at 25°C (at least 0.5 ml per ganglion) containing 10 µM amino-oxyacetic acid (AOAA) to inhibit transamination of accumulated GABA and 0.1 µM [^{3}H]-GABA. After 30 min the ganglia were removed, rinsed briefly (about 1 s) in non-radioactive solution, weighed and tissue radioactivity measured as described previously (Bowery & Brown, 1972). Inhibitors were added simultaneously with [^{3}H]-GABA. The concentration of [^{3}H]-GABA in the tissue was calculated in terms of the specific activity of [^{3}H]-GABA in the surrounding medium, as mmol/kg wet wt, and divided by the concentration in the medium (mmol/l), to yield a tissue/medium ratio. Saturable, carrier-mediated influx was calculated after subtracting the tissue/medium ratio (0.7) obtained at 5 mM external GABA to correct for the small (~5%) component of apparently non-saturable uptake (Bowery, 1974). Tritium retained in the presence of AOAA was $>95\%$ unchanged [^{3}H]-GABA (Bowery & Brown, 1972; Walsh, Bowery, Brown & Clark, 1974). Uptake is linear with time to 30 min, so provides an approximation to the initial influx velocity.

Efflux of GABA

Ganglia were incubated in [^{3}H]-GABA solution and then superfused with non-radioactive solution at a constant rate of 0.5 ml/minute. Both incubation and superfusion fluids contained 10 µM AOAA, such that effluent ^{3}H was unchanged [^{3}H]-GABA (see below).

Effluent superfusate was collected at 2 min intervals and its radioactivity measured. After completing the period of superfusion the residual radioactivity in the ganglion and nerve trunks was also determined. The rate of effluent radioactivity could then be expressed as the efflux rate coefficient, that is, the amount of radioactivity collected during a 2 min period divided by the arithmetic mean of the total radioactivity present in the tissue at the beginning and end of the same collection period. The latter was found by cumulatively summing the effluent radioactivity from the end of that collection period onwards with the residual radioactivity in the ganglion. Since the rate coefficient (k) so measured is very small compared with the collection period and with the rate of clearance from the extracellular space (see Brown & Scholfield, 1974) it approximates closely to that given by the differential equation

$$k = -\frac{dC_{\text{in}}}{dt} \cdot (C_{\text{in}})^{-1}\ (\text{min}^{-1})$$

assuming single compartment kinetics, where C_{in} is the intracellular GABA concentration. Contamination of effluent [^{3}H]-GABA by tritiated metabolites was assessed in separate control experiments. Several ganglia were incubated for 4 h in 5 μM [^{14}C]-GABA in the presence of AOAA and then incubated in 4 ml Krebs solution at 25°C (bubbled continuously with 95% O_2 and 5% CO_2) for up to 17 hours. The incubation fluid was concentrated by evaporation, solutes redissolved in 200 μl de-ionized water and subjected to paper electrophoresis (Walsh *et al.*, 1974). When AOAA (10 μM) was present in the effluent medium, >98% of radioactivity was identified as [^{3}H]-GABA. Hence, we assume that measured radioactivity during experimental runs also refers to unchanged [^{3}H]-GABA.

Total GABA

In a few experiments endogenous concentrations of GABA and several other amino acids, and net changes in GABA levels following incubation in GABA solution, were measured by a dansylation method. After an appropriate period of incubation, individual ganglia were weighed and homogenized in 100 μl of distilled water containing a known number of d/min of ^{14}C-labelled aspartate, glutamate, glutamine, glycine, alanine and GABA and [^{35}S]-taurine as internal standards. An equal volume of acetone was then added and the mixture evaporated to dryness by blowing compressed air over the surface of each sample. The amino acids were dissolved in 7 μl of a solution of sodium hydroxide (pH 11.3) and reacted with [^{3}H]-5-dimethylaminonaphthalene-sulphonyl chloride ([^{3}H]-dansyl chloride) at 40°C in the dark for 30 min (Briel & Neuhoff, 1972). The radioactive dansyl-amino acids were separated from each other and from contaminants by thin layer chromatography (Joseph & Halliday, 1975) and the radioactivity estimated by scintillation spectrometry. The yield of each dansyl-amino acid was ascertained by estimating the recovery of the internal standards and results have been expressed as mmol/kg wet tissue weight.

Autoradiographs

Ganglia were incubated in [^{3}H]-GABA solution with added AOAA, rinsed for 60 min in non-radioactive solution, fixed with glutaraldehyde, embedded in paraffin, sectioned at 5 μm and autoradiographs prepared as described by Young *et al.* (1973). Glutaraldehyde binds 30–60% of accumulated [^{3}H]-GABA; the retained label shows a distribution pattern comparable to that obtained using freeze-dried frozen sections (see Young *et al.*, 1973).

Depolarization

Ganglion cell responses to GABA and analogues were monitored as surface-depolarization recorded as described by Bowery & Brown (1974) with a modification for continuous superfusion (Brown & Marsh, 1975). Potential changes were recorded continuously with time on a potentiometric chart recorder. Agonists were applied by superfusion for 4 min at 20–30 min intervals, to minimize desensitization.

Compounds

[^{3}H-2,3]-γ-aminobutyric acid (10 Ci/mmol) was obtained from New England Nuclear. Purity (>95%) was checked as described previously (Bowery & Brown, 1972). [^{3}H]-dansyl chloride (8 Ci/mmol, uniformly labelled) was obtained from New England Nuclear and ^{14}C- and ^{35}S-labelled amino acids were obtained from the Radiochemical Centre. Unlabelled compounds were obtained as follows: γ-aminobutyric acid (4-amino-*n*-butyric acid, GABA), BDH; β-alanine (3-aminopropropionic acid, BALA), BDH; DL-β-amino-*n*-butyric acid (DL-3-aminobutanoic acid, β-aminobutyric acid, BABA), Sigma; 3-aminopropanesulphonic acid (3-APS), K & K Laboratories. Methylbicuculline was synthesized by J.F. Collins according to the method of Johnston, Beart, Curtis, Game, McCulloch & MacLachlan (1972).

Results

The results are divided into two sections. Part A concerns interaction of β-alanine (BALA) and β-amino-*n*-butyric acid (BABA) with the inward glial-cell carrier for GABA, as determined by their effects on the influx and efflux of [^{3}H]-GABA; part B concerns the depolarization of the neurones by BALA

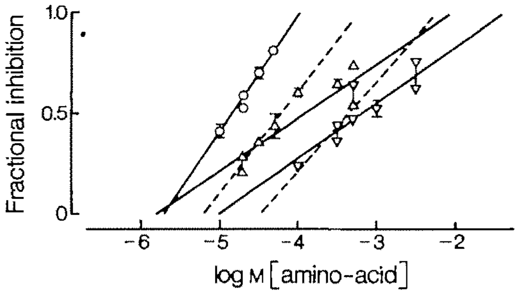

Figure 1 Effect of unlabelled γ-aminobutyric acid (GABA, ○), β-alanine (BALA, △) and β-amino-n-butyric acid (BABA, ▽) on the influx of [^{3}H]-GABA into rat isolated superior cervical ganglia. Ganglia were incubated for 30 min at 25°C in 0.5 μM [^{3}H]-GABA in the presence of 10 μM amino-oxyacetic acid, with or without unlabelled amino acid. Tissue concentrations of [^{3}H]-GABA were corrected for infiltration of the extracellular space and for non-saturable cellular uptake (see Methods section) to determine the saturable influx rate. Influx rate in the presence of unlabelled amino acid was expressed as a fraction of pooled controls (n=26), to give fractional inhibition of uptake (ordinates). Abscissa scale gives the log molar concentration of unlabelled amino acid. Points show single determinations or means ± s.e. (bars) where $n \nless 3$. Lines are least-squares regression coefficients for the full range of amino acid concentrations (———) or for concentrations corresponding to fractional inhibition $\ngtr 0.6$ (- - - -, see text).

and BABA and its variation under conditions where the internal GABA concentration is changed or their interaction with the carrier is suppressed.

Part A

Transport of GABA

BALA and BABA inhibited the influx of GABA and accelerated its efflux.

1. *Inhibition of [^{3}H]-GABA influx.* Figure 1 shows the auto-inhibition of [^{3}H]-GABA uptake by unlabelled external GABA and the hetero-inhibition of uptake by BALA (β-alanine) and BABA (β-aminobutyric acid). As expected, the slope of the least-squares regression line (0.58) for auto-inhibition of [^{3}H]-GABA influx accords with the maximum slope (0.59) predicted for competitive inhibition in a monomolecular substrate-carrier reaction (see, for example, Goldstein, Aranow & Kalman, 1968). The regression slopes for hetero-inhibition are much less. The probable reason for this is that hetero-inhibition was incomplete, in the sense that the amount of [^{3}H]-GABA accumulated was reduced by only 70–80% even at very high (5 mM) inhibitor concentrations. (By definition, see Methods section, auto-inhibition of saturable uptake was complete at 5 mM GABA.) On restricting data analysis to the effects of low inhibitor concentrations ($\ngtr$ 100 μM BALA, $\ngtr$ 300 μM BABA) the regression slopes (0.51 and 0.45 respectively, interrupted lines in Figure 1) approached more closely those for competitive hetero-inhibition.

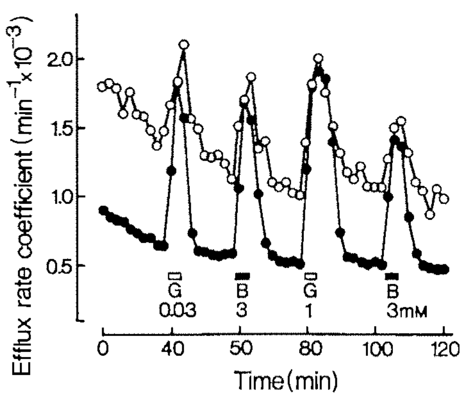

Figure 2 Effects of 4 min applications of unlabelled γ-aminobutyric acid (G) and β-amino-n-butyric acid (B) on the rate coefficient for efflux of tritium from paired rat superior cervical ganglia previously incubated in 1 μM [^{3}H]-GABA for 180 min (●) or 1 mM [^{3}H]-GABA for 60 min (○). All solutions contained 10 μM amino-oxyacetic acid. Ordinates: rate coefficient (min^{-1} × 10^{-3}). Abscissae: time after completing pre-incubation in [^{3}H]-GABA (min).

From these modified regression lines, mean concentrations producing 50% inhibition (IC$_{50}$) were: GABA, 14.3 μM; BALA, 59.2 μM; and BABA, 423.5 μM. For competitive inhibition $IC_{50} = K_i(1 + S/K_s)$. where K_i is the equilibrium dissociation constant of the inhibitor-carrier complex, S is the substrate (GABA) concentration, and K_s is the equilibrium dissociation constant of the substrate-carrier complex. Since S (0.1 μM) is much less than K_s (=K_m, 7 μM: Bowery & Brown, 1972), these IC$_{50}$ values should approximate to K_i.

2. *Acceleration of [^{3}H]-GABA efflux.* When ganglia were pre-incubated in [^{3}H]-GABA (1 μM for 180 min) and then superfused with non-radioactive Krebs solution, addition of unlabelled GABA, BALA or BABA to the superfusion fluid increased the rate at which tritium appeared in the effluent superfusate (see Figures 2 and 4). The augmented efflux rate coefficient attained a maximum level 4 min after adding the amino acid, remained elevated for up to 60 min during the continued presence of the amino acid, and then subsided to the control level within 10 min of

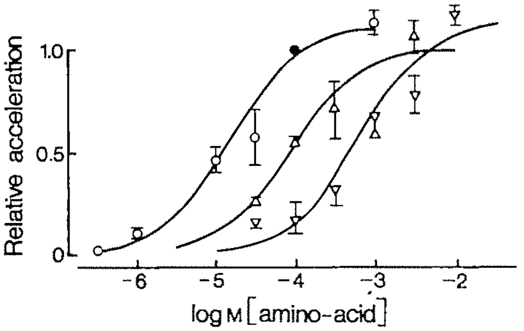

Figure 3 Mean dose-response curves for the acceleration of [^{3}H]-γ-aminobutyric acid (GABA) efflux produced by unlabelled GABA (○), β-alanine (BALA, △) and β-amino-n-butyric acid (BABA, ▽) determined from 6 experiments of the type shown in Figure 2. In each experiment a series of concentrations of GABA and either BALA (3 experiments) or BABA (3 experiments) were applied, and the increment in rate coefficient was calculated as $\Delta k/k^{\mathrm{rest}}$, where Δk is the difference between the rate coefficients before and during application of amino acid and k^{rest} is the resting rate coefficient (see text). Ordinates show this increment expressed as a fraction ($=f'$) of that produced by 100 µM GABA (●). Points are means ± s.e. ($n=6$ for GABA, 3 each for BALA and BABA). Curves are drawn according to the hyperbolic expression $f'=f'_{\mathrm{max}}S/(K_E+S)$ where S is the amino acid concentration and f_{max} and K_E are constants. Values for f_{max} and K_E were derived from least-squares regressions for the linear transformation $f'=f'_{\mathrm{max}}-K_E(f'/S)$ (Dowd & Riggs, 1965). The nature of the curve is discussed further in the Appendix.

removing the amino acid. With 4 min exposure periods, the effect of a single concentration of an amino acid could be repeated fairly consistently if applied at 20 min intervals.

Concentration-dependence The acceleration of [^{3}H]-GABA release increased with augmenting concentrations of external amino acid in the manner shown in Figure 3. In these experiments a random series of concentrations of unlabelled amino acid was administered with a contact time of 4 min and dose-intervals of 20 min, starting 45 min after the completion of the preincubation period, when the efflux rate coefficient had descended to a fairly steady value (mean $k^{\mathrm{rest}}=0.64\pm0.05\times10^{-3}$ min^{-1}). The peak rate coefficient (k') attained in the presence of each concentration (S) of amino acid was expressed as a multiple of the immediately-preceding resting value, and the increment expressed in the form $(k'/k^{\mathrm{rest}})-1$ $(=\Delta k)$. All such increments were expressed as a fraction (f') of that produced by 100 µM GABA, to minimize variations between preparations.

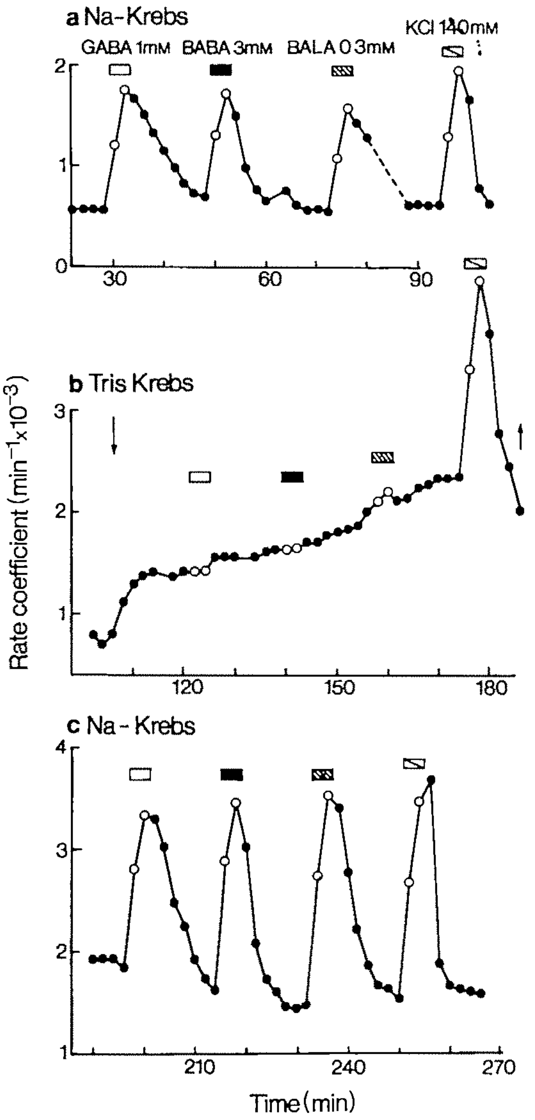

Figure 4 Effect of replacing external Na$^+$ ion with TRIS on the acceleration of [^{3}H]-γ-aminobutyric acid (GABA) efflux produced by GABA (1 mM), β-amino-n-butyric acid (BABA, 3 mM), β-alanine (BALA, 0.3 mM) and KCl (140 mM, added to solution). (a), (b) and (c) represent continuous time records of the efflux rate coefficient k (min^{-1} x 10^{-3}, ordinates) into: (a) and (c) normal Krebs solution; and (b) Na$^+$-free, 150 mM TRIS solution (substituted and removed at the arrows). All solutions contained 10 µM aminooxyacetic acid. The ganglion had been previously incubated for 180 min in 1 µM [^{3}H]-GABA. Temperature, 25°C. (During this rather prolonged exposure to TRIS solution the rate coefficient showed a progressive increase after an initial rise and did not return to its control level on restoring Na$^+$: responses to shorter exposures were fully reversible.)

The fractional increments varied as a hyperbolic function of S such that $f'/f'_{\mathrm{max}} = S/(S+K_E)$, where K_E is the concentration of amino acid producing a half-maximal acceleration of efflux (see Appendix). Regression analysis of a least-squares linear transform (see legend to Figure 3) yielded values for K_E of 16, 85 and 606 µM for GABA, BALA and BABA respectively (Table 1). A fourth amino acid with a much more pronounced depolarizing action, 3-aminopropanesulphonic acid (3-APS, see Bowery & Brown, 1974) showed a K_E of 341 µM (Table 1, omitted from Figure 3 for clarity). All four amino acids produced comparable maximal increments in the fractional efflux rate coefficients f_{max}: when reconverted to absolute rate coefficients these ranged from means of 2.05 to 2.30 × 10^{-3} min^{-1}, a 3.2 to 3.6-fold increase over the mean resting rate coefficient. The mean tissue ^{3}H concentration at the end of the pre-loading period in 1 µM [^{3}H]-GABA was 19.5 ± 3.8 µmol/kg tissue, representing some 9.5% labelling of the endogenous GABA (see Table 2 below).

External Na$^+$ ions. Replacement of external Na$^+$ with TRIS produced a sustained increase in the efflux rate coefficient, comparable to that produced by a high concentration of an amino acid (Figure 4). Subsequent addition of amino acid (in TRIS solution) then failed to accelerate the efflux any further, but addition of K$^+$ continued to stimulate efflux in TRIS solution as in normal Na$^+$ solution (see Bowery & Brown, 1972). Both the resting rate coefficients and the increments on adding an amino acid were fully restored within 20 min of restoring normal Na$^+$, providing the exposure time to TRIS did not exceed 30 minutes.

Table 1 Kinetic constants for the acceleration of [^{3}H]-γ-aminobutyric acid (GABA) efflux by unlabelled GABA, β-alanine (BALA), β-amino-*n*-butyric acid (BABA) and 3-aminopropanesulphonic acid (3-APS)

	K_E (µM)	k_{max} (min^{-1} × 10^{-3})
GABA	16.0 ± 4.4	2.27 ± 0.09
BALA	85.3 ± 21.5	2.10 ± 0.09
3-APS	341 ± 64	2.05 ± 0.08
BABA	606 ± 115	2.30 ± 0.10

Constants (means ± s.e.) were derived from least-squares regression lines for the linear expression $f = f_{\mathrm{max}} - K_E\ (f/S)$ (see legend to Figure 3) where f is the normalized fractional increment in efflux rate coefficient in the presence of amino acid at concentration S, f_{max} is the maximal increment at $S = \infty$, and K_E is the concentration of amino acid producing half-maximal acceleration in efflux rate. The extrapolated true maximal rate coefficient k_{max} (min^{-1}) was calculated from f_{max} and the average resting rate coefficient (= 0.635 × 10^{-3} min^{-1}).

Part B

Depolarization

The objective of these experiments was to find out if the accelerated efflux of GABA produced by BABA or BALA, as described above, led to a depolarization of the ganglionic neurones. This required some means of distinguishing between a depolarization produced by the release of GABA and that resulting from a direct action of the amino acids on the neuronal GABA-receptors. Two approaches were attempted: (1) to increase the amount of GABA available for release, so as to exaggerate indirect effects mediated through such release; and (2) to inhibit amino acid-driven exit.

1. *Effect of increasing endogenous GABA* Ideally, it would be more helpful to remove endogenous GABA and thereby eliminate the possibility of its release. However, we have so far been unable to devise a satisfactory method for depleting the tissue of GABA, and so have been forced to adopt the more indirect approach of artificially increasing ganglionic GABA levels. This may be readily achieved by incubating the tissue in a high concentration of exogenous GABA.

Table 2 Effect of incubating ganglia* in 1 mM γ-aminobutyric acid (GABA) for 60 min in the absence (*a*) and presence (*b*) of 10 µM amino-oxyacetic acid (AOAA) on their content of GABA

		a *−AOAA*	*b* *+AOAA*
(1)	−GABA	0.153 ± 0.017 (1.21)	0.205 ± 0.015 (1.63)
(2)	+GABA	0.318 ± 0.031 (2.52)	0.729 ± 0.053 (5.79)
(3)	+GABA −GABA	2.08 ± 0.13	3.56 ± 0.14

Numbers give measured GABA concentrations (mean ± s.e., $n = 4$) expressed as mmol/kg wet weight; figures in brackets are calculated concentrations of GABA in the neuroglial cells, based on the premises that (i) GABA is confined to glial cells and (ii) glial cells comprise 13% of tissue volume (D.L. Tamarind, personal communication from examination of electron micrographs). Ratios +GABA/−GABA are mean ratios measured in paired contralateral ganglia from the same rats (mean ± s.e., $n = 4$).

* Ganglia were incubated overnight at 4°C in Krebs solution, pre-equilibrated for 3 h in Krebs solution at 25°C (with or without AOAA), incubated for a further 60 min in the absence or presence of 1 mM GABA, washed for 30 min in Krebs solution, homogenized and amino acid levels determined by micro-dansylation (see Methods section).

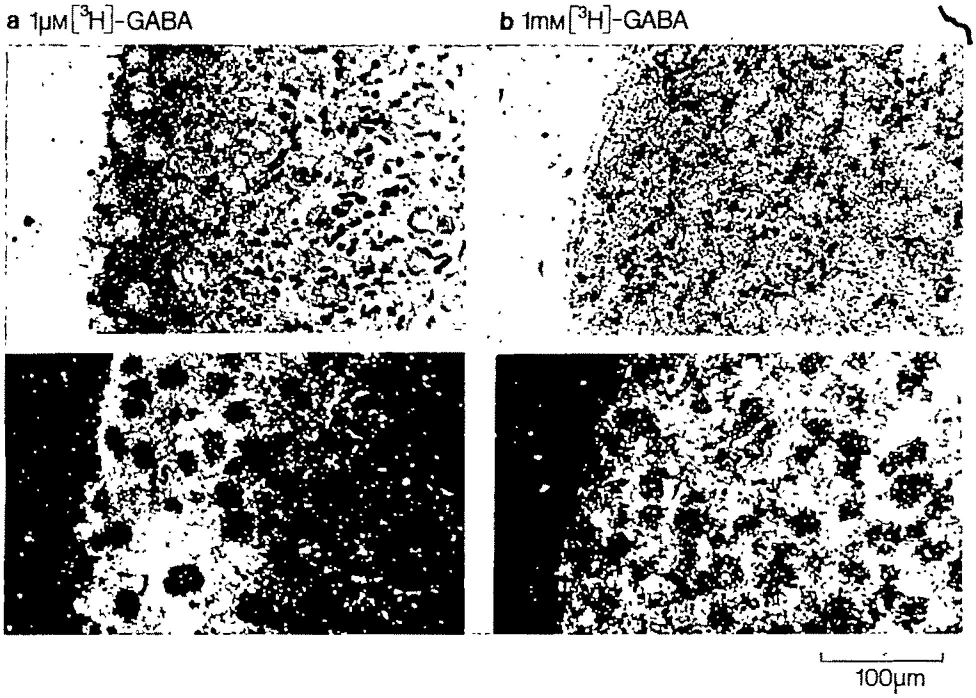

Figure 5 Autoradiographs of sections of rat superior cervical ganglia previously incubated for 60 min in (a) 1 μM [^{3}H]-γ-aminobutyric acid (GABA) and (b) 1 μM [^{3}H]-GABA with 1 mM unlabelled GABA added. Upper photographs were taken under transmitted light, lower photographs (of the same field) under incident light. Exposure times: 28 days. Scale, 100 μm.

Thus, when ganglia were incubated in 1 mM GABA for 60 min at 25 °C the total tissue level was increased by 108% as measured in paired contralateral ganglia, from 0.15 ± 0.02 to 0.32 ± 0.03 mmol/kg tissue (Table 2). When metabolism of GABA was inhibited throughout with 10 μM amino-oxyacetic acid (AOAA), a much greater increase of 256% in GABA concentration occurred following such incubation, from 0.21 ± 0.02 to 0.73 ± 0.05 mmol/kg tissue. Further analysis between paired ganglia confirmed that addition of AOAA alone (without added GABA) might produce an increase of some 55 (±20)% in GABA levels from 0.16 to 0.23 mmol/kg tissue. Addition of either GABA or AOAA or both did not materially affect the tissue concentrations of the other amino acids measured. Average concentrations of these were mmol/kg wet weight, mean ± s.e., $n = 24$): glycine, 2.58 ± 0.16; taurine, 3.14 ± 0.20; α-alanine, 1.40 ± 0.07; aspartate, 2.51 ± 0.12; glutamate, 4.85 ± 0.26; glutamine, 1.36 ± 0.10. (It should be pointed out that these values do not necessarily represent fresh tissue levels, but rather those obtaining under the experimental conditions used to measure depolarization, in which the ganglia were first incubated overnight at 4 °C (see below)).

When the incubation fluid contained 1 mM [^{3}H]-GABA the tissue [^{3}H]-GABA concentrations, expressed at the specific activity of the incubation medium, were 0.15 ± 0.02 and 0.59 ± 0.05 mmol/kg tissue in the absence and presence of AOAA respectively (mean ± s.e., $n = 4$ in each case). These values correspond quite well with the increments in total GABA in Table 2, suggesting that the bulk of accumulated [^{3}H]-GABA represents net uptake rather than exchange for endogenous GABA.

Autoradiographs (Figure 5) indicated this accumulated radioactivity to be extraneuronal in location. The cellular distribution pattern resembles that observed after incubating in a much lower (1 μM) concentration of [^{3}H]-GABA, which has been previously judged (from simultaneous electron microscope autoradiographs) to represent accumulation in neuroglial cells (see Young *et al.*, 1973).

Resting efflux rate coefficients tended to be rather greater in ganglia previously incubated in 1 mM GABA than in 1 μM GABA (see Figure 2). In 4 pairs of ganglia compared directly in this manner, mean resting rate coefficients ($\text{min}^{-1} \times 10^{-3}$, ± s.e.) were: (a) 1 μM [^{3}H]-GABA, 0.73 ± 0.10; (b) 1 mM [^{3}H]-GABA, 1.09 ± 0.07; b/a, 1.47 ± 0.19. (The rate coefficient

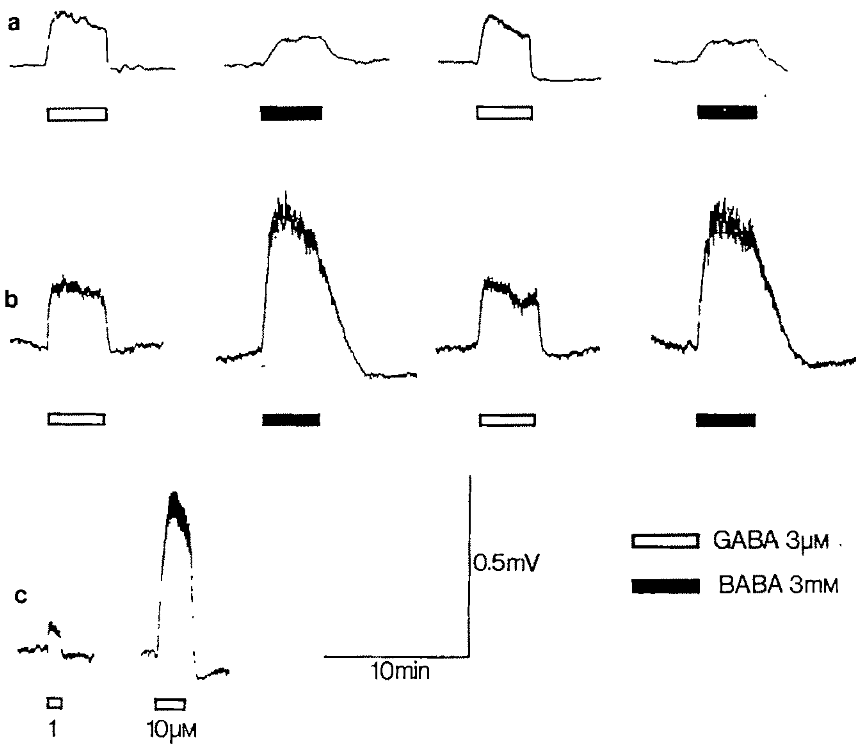

Figure 6 Records of surface depolarizations in a rat isolated superior cervical ganglion produced by 4 min applications of 3 μM γ-aminobutyric acid (GABA, open bars) and 3 mM β-amino-*n*-butyric acid (BABA, closed bars) (a) before, and (b) and (c) after superfusing with 1 mM GABA for 60 min, followed by 60 min washing. All solutions contained amino-oxyacetic acid (10 μM). Amino acids were applied at 30 min intervals. Calibrating GABA doses (c) were given at the end of the experiment for 1 min periods. Scales: 0.5 mV, 10 min.

tends to diminish with time, more rapidly at higher loading concentrations, so the mean values taken through a prolonged efflux run are not strictly comparable; initial maximum values may have more theoretical significance, but cannot be measured in practice: see Appendix.) More importantly, increasing the loading concentration does not alter the peak rate coefficient attained in the presence of a high concentration of GABA or BABA (see Figure 2).

Depolarization after increasing endogenous GABA levels. Figure 6 illustrates the effect of increasing endogenous GABA concentrations, by incubating the ganglion in 1 mM external GABA, on the depolarizing actions of BABA and GABA itself. It also serves to indicate the experimental design used in subsequent experiments of this type, described below. First, responses to 2–4 applications of BABA and GABA were recorded in a ganglion which had not been previously exposed to GABA but which had been incubated overnight at 4°C. The standard concentration of BABA used, 3 mM, was such as to produce a substantial acceleration of GABA efflux (see Figure 3). Initially, this concentration produced a very low amplitude depolarization, and was matched (approximately) by the standard concentration of 3 μM GABA (i.e. a just-suprathreshold concentration, well below the ED_{50} value of 12.5 μM, cf. Bowery & Brown, 1974). (The very low amplitude responses were, of course, helpful in detecting increments after loading, but necessitated very stable recording conditions; overnight incubation facilitated this, by allowing the initial 'injury potential' to dissipate.) The agonists were applied alternately for 4 min periods at 30 min intervals: the exposure time corresponded to that required for a maximal increment in measured [^{3}H]-GABA release, and the interval was sufficient to prevent desensitization of the response to the subsequent dose of agonist. After obtaining stable control responses, the ganglion was exposed to 1 mM GABA solution for 60 min, washed in GABA-free solution for a further 60 min, and the agonists re-applied. Finally, responses to other concentrations of GABA were usually obtained, so that the responses to

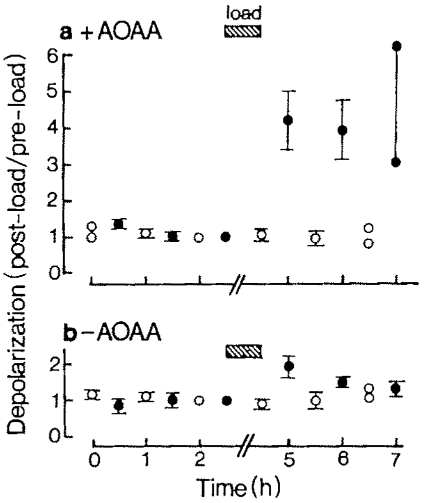

Figure 7 Effect of loading with γ-aminobutyric acid (GABA) (by superfusing with 1 mM GABA solution for 60 min) on depolarizations produced by 3 µM GABA (O) and 3 mM β-amino-*n*-butyric acid (BABA, ●). Each point gives the mean and s.e. mean of 4 experiments in the presence (upper graph) and absence (lower graph) of amino-oxyacetic acid (AOAA) in the superfusing solution. Depolarization amplitudes are expressed as a fraction of those produced by each agonist immediately prior to loading.

BABA could be approximately calibrated in terms of the equally-effective concentration of GABA. (This procedure was not satisfactory when the GABA dose-equivalents were less than 1–3 µM, since this is at the extreme lower limit of the GABA log dose–response curve.)

In the experiment illustrated in Figure 6 (performed in the continuous presence of AOAA) incubation in 1 mM GABA clearly increased the effect of BABA without producing any corresponding change in the response to 3 µM GABA. This differential effect was a consistent observation, as shown in Figure 7. An enhanced response to BABA was also consistently observed in the absence of AOAA, though of much smaller voltage-amplitude (Figure 7b).

Calibrated in terms of the responses to GABA, the effect of 3 mM BABA corresponded to that produced by between 1 and 4 µM GABA under control (pre-loading) conditions. Following loading the increased response corresponded to an additional 2 to 8 µM GABA in the presence of AOAA and <2 µM in the absence of AOAA (the lower amplitude responses here precluding accurate calibration). (In this respect Figure 6 illustrates a rather extreme increment, cf. Figure 8.)

The relative amplitudes of the depolarizations produced by the standard concentrations of 3 mM BABA and 3 µM GABA prior to loading varied appreciably between experiments, from 0.3 to 1.5 ($n=8$). This ratio showed no consistent difference in ganglia incubated in the presence or absence of AOAA. Further, addition of AOAA without added GABA during the course of an experiment did not enhance the effect of BABA (3 experiments).

Antagonism The depolarization produced by BABA after loading was blocked *pari-passu* that by GABA with methylbicuculline, indicating an effect solely upon GABA-receptors (cf. Bowery & Brown, 1974; Bowery, Brown & Collins, 1975).

β-Alanine BALA also accelerates the release of [^{3}H]-GABA (Figure 2) and, like BABA, showed an increased depolarizing action after loading in 1 mM GABA (3 experiments). An example is illustrated in Figure 8, using a standard concentration of 0.3 mM BALA. Here the increment in response to BALA was equivalent to an additional 1.3 µM GABA, as against 2.2 µM GABA for the increment in BABA response.

3-Aminopropanesulphonic acid (3-APS) This compound is a very potent GABA-receptor agonist, about 3 times more potent than GABA (Curtis, Phillis & Watkins, 1961; Bowery & Brown, 1974). Thus, 1 µM 3-APS produced a depolarization as great as 3 µM GABA. At such concentrations, 3-APS produces negligible acceleration of [^{3}H]-GABA efflux (EC$_{50}$, 341 µM; Table 1). Hence, if the increased depolarization produced by BABA or BALA after loading the ganglion with GABA was due to an increased release of GABA rather than to some change in receptor sensitivity, no such augmentation of 3-APS effects would be expected: this was verified in 3 ganglia, all showing the previously-described augmentation of BABA responses.

Dose-response curves A further attempt to quantitate the post-loading increases in depolarization was made from dose-response curves to GABA-mimetics. The dose-response curves for GABA and 3-APS were not materially altered by prior incubation with 1 mM GABA, except for a slight (not significant) reduction at very high response levels (Figures 9a and b). In contrast, curves for BABA and BALA show a significant sensitization at the lower end, with convergence or desensitization at the upper extremity (Figures 9c and d; although the curve for BABA is restricted to concentrations $\leqslant$30 mM, it shows the same tendency to converge at the higher response levels as that to BALA). This type of shift is contrary to that expected for receptor sensitization, but accords with that predicted for a limited increment in GABA produced by a release process. The increments in interstitial GABA required to account for the shift could be estimated by calibrating the responses to

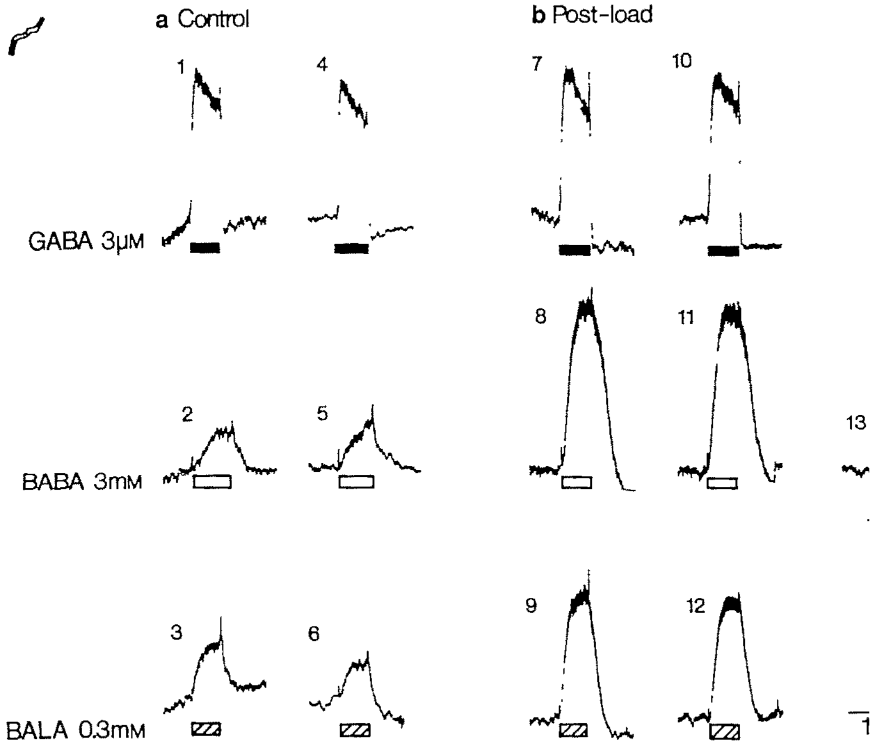

Figure 8 Depolarizing responses to γ-aminobutyric acid (GABA, 3 μM) β-amino-*n*-butyric a and β-alanine (BALA, 0.3 mM) (above downwards) recorded (a) before and (b) after loading f GABA (amino-oxyacetic acid present). Agonists were added for 4 min periods at 30 min sequence indicated. Responses to final calibrating doses of GABA are shown on the extreme 0.2 mV, 10 min. (Numbers indicate order of responses.)

BABA and BALA in terms of the equivalent concentrations of GABA, derived from the GABA dose-response curves. Results showed a maximum increment at carrier-saturating concentrations of the two agonists (cf. Figure 3) of about 2–3 μM interstitial GABA.

2. *Effect of inhibiting the GABA-carrier* One method of inhibiting the substrate-stimulated release of [^{3}H]-GABA is to remove external Na$^+$ ions, by replacement with TRIS or Li$^+$ (see Figure 4 above). Since the depolarizing action of GABA itself is not dependent upon Na$^+$ ions (Bowery, 1974; Adams & Brown, 1975), it appeared feasible to use Na$^+$-replacement as a means of eliminating indirect GABA-releasing effects. Unfortunately replacing external Na$^+$ caused very large shifts in the standing potential difference between the two recording electrodes, presumably reflecting differential rates of change of the liquid junction potentials. Further, after some time (60 min or more) in TRIS solution responses to GABA itself became attenuated, a feature previously observed using
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Discussion

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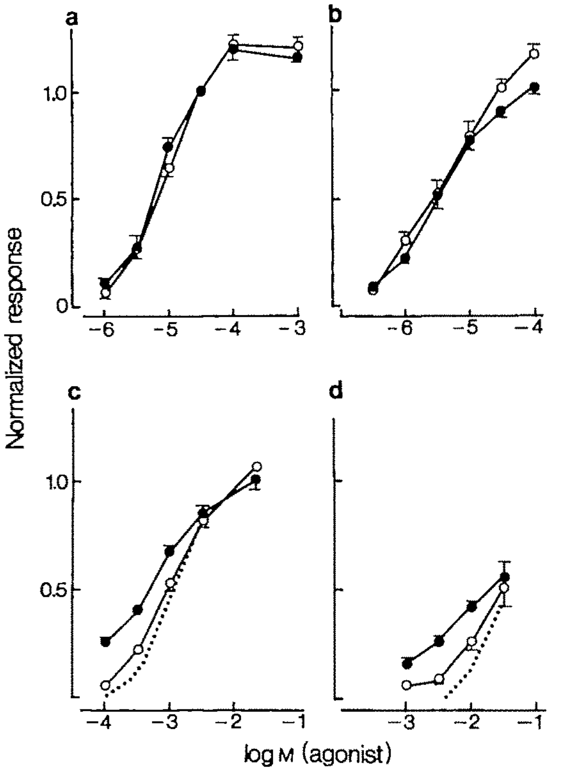

Figure 9 Dose-response curves to (a) γ-aminobutyric acid (GABA), (b) 3-aminopropanesulphonic acid (3-APS), (c) β-alanine (BALA) and (d) β-amino-n-butyric acid (BABA) measured before (○) or after (●) incubation in 1 mM GABA (amino-oxyacetic acid present throughout). Responses were normalized with respect to those produced by 30 μM GABA (=1%) to permit comparison between different preparations. Each point is the mean of 4 ganglia; bars=s.e. mean. The dotted lines (......) in (c) and (d) show calculated responses in a tissue totally depleted of GABA (i.e. in the absence of GABA-release), based on calculated interstitial GABA concentrations in Figure 12.

concentration of GABA. Our interpretation of this is that BABA and BALA release GABA from the neuroglial cells into the interstitial spaces, and that the depolarization of the ganglion cells which they produce results (in low doses at least) from the action of the released GABA rather than from their direct action on the neuronal GABA-receptors. The increase in the depolarization after exposure to GABA then results from the accumulation of GABA in the glial cells: extra GABA is therefore available for release.

The principal lines of experimental evidence supporting this interpretation may be summarized as follows:

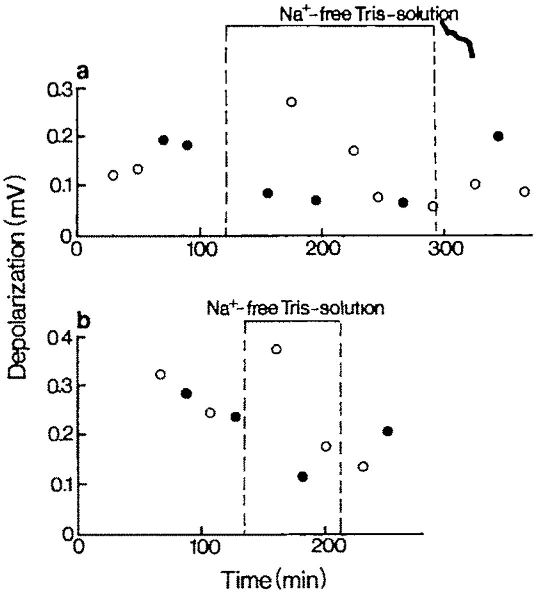

Figure 10 Effects of replacing external Na^+ ions with TRIS on the depolarizing responses of two rat isolated ganglia to 3 μM γ-aminobutyric acid (GABA, ○) and 3 mM β-amino-n-butyric acid (BABA, ●), (a) without prior incubation in GABA and (b) after incubation for 60 min in 1 mM GABA. (Amino-oxyacetic acid present.) Ordinates: mV peak depolarization; abscissae: time from first test responses.

(a) In concentrations required to depolarize the ganglion both BABA and BALA produce a substantial acceleration of [^{3}H]-GABA efflux (Table 3). In contrast, 3-APS, whose effect is not

Table 3 Comparison of the effects of amino acids on (a) γ-aminobutyric acid (GABA) receptors and (b) GABA efflux

	a	*b*	*c*
	Receptor ED_{50} (μM)	*Carrier K_E (μM)*	*a/b*
3-APS	3.7	341	0.011
GABA	12.5	16	0.78
BALA	1,300	85	15.24
BABA	>30,000	606	>50

Values in (a) are ED_{50} concentrations for the depolarization of isolated ganglia not previously exposed to GABA, deduced from the data of Bowery & Brown (1974) and Bowery (1974); values in (b) are the K_E values in Table 1 for acceleration of [^{3}H]-GABA efflux. BALA=β-alanine; BABA=β-amino-n-butyric acid; 3-APS=3-aminopropanesulphonic acid.

potentiated after GABA-loading, only accelerates GABA-efflux in concentrations well above those required to depolarize the neurones.

(b) The shift in the dose-response curves to BABA and BALA after loading accord with the effect of a limited increment in agonist (GABA) concentration, rather than a general receptor-sensitization.

(c) Potentiation was enhanced when metabolism of GABA was inhibited with amino-oxyacetic acid, according with the greater amount of GABA accumulated.

(d) The potentiated response to BABA was reduced in Na$^+$-free solution, which inhibits efflux-acceleration by BABA but does not itself prevent neuronal depolarization by GABA (see also Adams & Brown, 1975).

Mechanism of efflux acceleration

It seems clear from the relative abilities of the different amino acids employed in this study to inhibit [^{3}H]-GABA uptake and accelerate [^{3}H]-GABA efflux (compare Figures 1 and 2) that the latter arises through an interaction with the inward GABA-carrier. In fact, β-alanine appears to serve as a substrate for the glial GABA-carrier (Schon & Kelly, 1975; Bowery & Brown, unpublished observations), and one might suppose the same to apply to BABA.

There are essentially two ways in which a substrate for the inward carrier might accelerate GABA-efflux: accelerated exchange diffusion or inhibition of a continuous re-accumulation process. The former implies that GABA is transported in both directions across the cell membrane by the same carrier, but that the outward transport rate is normally submaximal; then addition of a substrate to the outside and its consequent inward transport may increase the rate of turnover of the carrier and thereby accelerate the exit rate (see, for example, Stein, 1967). With re-accumulation it is envisaged that GABA leaves the cells at a constant rate and that a proportion of the effluent GABA is then re-accumulated from the pericellular fluid by the inward carrier; occupation of the external carrier sites by another substrate (or by unlabelled substrate if the exit of labelled material is considered) will reduce the rate of re-accumulation and produce an increased overflow into the external medium (a 'leak-and-pump' situation: Willbrandt & Rosenberg, 1963).

In both cases the increased efflux rate depends, in the first instance, on the degree of saturation of the inward carrier, and hence will show a similar dependence upon the external substrate concentration. On other grounds, however, we tend to view a reuptake-inhibition process as the most likely system responsible for efflux acceleration in the ganglion. Two pieces of experimental information favour this. First, the efflux rate is also increased in Na$^+$-free solution to a comparable extent to that attained on saturating the external carrier (Figure 4): Na$^+$-free solution inhibits inward transport and would slow the carrier turnover rate rather than accelerate it. Second, unless the internal face of the carrier was very unsaturated increasing the intracellular GABA concentration should reduce the rate coefficient for carrier-mediated exit of GABA, whereas the opposite tendency (if anything) occurred (see Results section and Figure 2). This suggests that the exit process *per se* tends to follow diffusion kinetics, i.e. is governed by a constant rate coefficient, independent of intracellular concentration. (This need not imply that exit is due to true diffusion; more likely, exit is mediated through a carrier whose Michaelis constant is sufficiently in excess of the internal GABA concentration as to render the rate of exit directly proportional to $[\text{GABA}]_{\text{in}}$ within the narrow range of experimental variation in $[\text{GABA}]_{\text{in}}$ exployed in our experiments.)

Quantitative considerations

In the Appendix some estimates have been made of the increments in interstitial GABA concentration expected to accompany accelerated efflux of intracellular GABA. These calculations are based on the above mechanistic interpretation that the accelerated efflux of GABA results from inhibition of re-accumulation, but would, in fact, also be valid for the alternate situation where re-accumulation was negligible and efflux was increased by accelerated exchange diffusion. The results predict that carrier-saturating concentrations of BABA or BALA would increase the interstitial GABA concentration by 1.04 μM in the resting (unloaded) state and by 3.41 μM if the ganglion had been previously loaded with GABA by pre-incubation in GABA solution as described above. The potentiation would therefore correspond to an additional 2.37 μM interstitial GABA. These values accord very closely with those (2–3 μM) estimated from the modification of the dose-response curves. Notwithstanding the many simplifications used in the calculations, this argument suggests that the concept is numerically reasonable, at the very least.

A corollary to this is that, even without prior loading with GABA, the depolarizing responses to low concentrations of GABA and BALA may be attributed entirely to the release of GABA. This results from the not-negligible resting intracellular GABA concentrations in the tissue. Thus, if the responses attributable to the calculated increments in interstitial GABA are subtracted, the dose-response curves for BABA and BALA are shifted further to the right (interrupted lines in Figure 9): this corrected curve would indicate the response attributed to direct receptor-activation. (It may be noted that the increment in interstitial [GABA] resulting from carrier-saturation is so low as not to affect materially the responses at which previous potency measurements were made (Bowery & Brown, 1974): these latter would reflect solely the direct action of the analogues on the neural receptors.) Clearly, it would be helpful to obtain experimental corroboration of this indirect component by depleting the tissue of its normal GABA levels. This we have so far been unable to achieve. However, some support is given by the observation that the response to low BABA concentrations in normal (unloaded) ganglia is reduced in Na^+-free solution (Figure 10).

Implications

The indirect GABA-mimetic action described in this paper is not likely to be an important mechanism of drug action on sympathetic ganglia *per se* since, while detectable in terms of depolarization, the amount of GABA released is too low to affect transmission (see De Groat, 1970; Adams & Brown, 1975). Instead the experiments might be viewed as suggesting a possible mechanism of drug action in the brain, where GABA fulfils the role of a natural inhibitory neurotransmitter. In this context the following considerations may be relevant:

(a) The endogenous GABA concentrations in the brain exceed those attained in ganglia, even after the latter have been enhanced by prior loading. Maximal values quoted include 9.7 mmol/kg (Fahn & Coté, 1968) and 4.3 mmol/kg (Balcom, Lenox & Meyerhoff, 1975), i.e. 5–10 times those attained in ganglia (cf. Table 2).

(b) Addition of carrier-substrates has been observed to accelerate the efflux of GABA from central nervous system preparations (Cutler, Hammerstad, Cornick & Murray, 1971; de Belleroche & Bradford, 1972; Simon, Martin & Kroll, 1974; Levi & Raiteri, 1974; Raiteri, Federico, Colethi & Levi 1975). When measured under comparable experimental conditions the rate coefficients for resting and substrate-stimulated efflux of [^{3}H]-GABA from isolated mammalian olfactory cortex slices are equal to or exceed those for the corresponding release from ganglia (Brown & Scholfield, unpublished observations). Taken in conjunction with (a), and assuming comparable clearance rates, this would imply that the interstitial GABA levels in brain attained during such release might exceed those in ganglia by an order of magnitude. (Substrate-stimulated release from brain has been attributed to accelerated exchange-diffusion, rather than inhibited reuptake; however, this would not affect calculations of interstitial GABA concentrations, see Appendix.)

(c) It seems reasonable to assume that the sensitivity of central neurones to GABA is no less than that of ganglionic neurones (see Adams & Brown, 1975). Further, for equivalent degrees of receptor-activation GABA would exert a more profound depressant action on central transmission by virtue of the neuronal hyperpolarization (cf. Dreifuss *et al.*, 1969 with Adams & Brown, 1975). Thus, if (from (a) and (b) above) a carrier-substrate raised interstitial GABA concentrations to 10–20 µM, appreciable inhibition of excitatory transmission would be anticipated.

Some complexity is introduced by the fact that, in the brain, GABA is present in both glial cells and inhibitory nerve terminals. The precise partition of endogenous GABA between these two loci is rather uncertain. Intra-terminal concentrations have been estimated at 60 mM (Fonnum, 1973) and 12.5 mM (Patel, Johnson & Balazs, 1974). The glial 'pool' may account for 1.3 mmol/kg brain (Patel *et al.*, 1974) or 0.9 mmol/kg pineal (Schon *et al.*, 1975); if corrected for the glial volume, a glial concentration of at least 5–10 mM seems probable. Both pools appear subject to substrate-stimulated release, but the substrate-specificities of the carriers differ. As in ganglia, BALA is an effective substrate for the glial carrier (Schon & Kelly, 1975; Schon *et al.*, 1975) and causes an accelerated efflux of [^{3}H]-GABA from olfactory cortical slices (Brown & Scholfield, unpublished observations). L-2,4-Diaminobutyric acid (DABA) is a good substrate for the synaptosomal (presumed neural) carrier (Iversen & Johnston, 1971; Simon & Martin, 1973; Kelly, Dick & Schon, 1975), and accelerates GABA-efflux from brain synaptosomes (de Belleroche & Bradford, 1972; Simon *et al.*, 1974). BABA has also been reported to accelerate the release of GABA from synaptosome preparations (de Belleroche & Bradford, 1972). BABA, DABA and BALA would therefore all be potential indirect GABA-mimetics in brain, albeit releasing GABA from different loci.

It may be difficult, at present, to design definitive tests for such indirect effects in brain. One rather oblique approach might be to seek marked discrepancies between the potencies of amino acids as agonists in brain and ganglia, on the basis that (i) the GABA-receptors are structurally similar at the two sites (Bowery & Brown, 1974) but that (ii) the endogenous glial concentration of GABA is probably lower, and neural GABA absent, in the ganglion. From this viewpoint it may be of significance that DABA has only 0.1% of the activity of GABA on ganglionic neurones (Bowery & Brown, 1974) but appears to be a relatively much stronger GABA-mimetic on central neurones (Purpura, Girado, Smith, Callan & Grundfest, 1959; Curtis & Watkins, 1960; Curtis *et al.*, 1961). It would be interesting to know whether the GABA-releasing action of DABA accounts for this difference.

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APPENDIX

KINETICS OF SUBSTRATE-STIMULATED EFFLUX

D.A. BROWN & L. SAUNDERS

We have described above how the addition of unlabelled GABA or certain analogues accelerates the efflux of labelled GABA from the ganglion. In this Appendix we consider further some quantitative aspects of this acceleration, from which to derive an estimate of the changes in interstitial GABA following application of a carrier-substrate.

For these calculations we have selected, as an operational model, the situation where the exit of GABA from the cells shows a simple first-order dependence upon internal GABA concentration (within the range of variation in the latter generated in the present experiments), but where the consequent interstitial concentration and final exit rate into the surrounding medium is modified by a continuous re-accumulation process mediated by a saturable inward carrier. BABA or BALA then act as alternative substrates for the inward carrier, and accelerate efflux primarily by saturating the inward carrier so as to inhibit re-accumulation of GABA. If exit of labelled GABA is considered, external unlabelled GABA exerts a similar inhibitory effect. Reasons for selecting this model, rather than an accelerated exchange diffusion system, are given in the Discussion section; however, as pointed out below, the numerical results are likely to be similar since in either case, the acceleration depends ultimately upon the degree of saturation of the inward carrier.

Analysis

The superfused ganglion may be considered to comprise three compartments in series—the intracellular compartment X, the interstitial fluid Y and the surrounding perfusion fluid Z. Throughout the efflux collection the net flow of intracellular GABA is in the direction $X \rightarrow Z$.

Let the volumes of the compartments (m^3) be V_x, V_y and V_z respectively; let the amounts of GABA (mol) in each compartment at time t be Q_x, Q_y and Q_z; and let the concentrations of GABA (mol m^{-3} = mM) at time t be x, y and z (Q_x/V_x, Q_y/V_y, Q_z/V_z).

Since $V_z \geqslant 1000$ (V_y, V_x) and Q_z is very small, $z \ll y$ at all values of t, so that diffusional backflux from $Z \rightarrow Y$ can be neglected.

Calculation is simplified (but not invalidated, see 'Limitations', below) if the intracellular GABA initially present in the tissue is regarded as being uniformly distributed throughout the intracellular fluid, since, in this case, $V_x \approx V_y = V$ (see Brown, Halliwell & Scholfield, 1971).

At $t = 0$ all of the GABA in the system is present in the intracellular compartment X. Then a conservation equation applies:

$$Q_x + Q_y + Q_z = Q_0$$

or

$$x \cdot V + y \cdot V + z \cdot V_z = x_0 V \tag{1}$$

where the subscript (0) refers to $t = 0$.

Transfer rates between compartments may be expressed as follows:

(i) *Efflux* X → Y: diffusive, governed by a single rate coefficient k_1 (see above):

$$-\frac{dx}{dt} = k_1 x \tag{2}$$

(ii) *Influx* Y → X: a saturable function of y

$$+\frac{dx}{dt} = \frac{\alpha y}{K + y} \tag{3}$$

where α and K are constants with units of velocity (mol m^{-3} min^{-1}) and concentration (mol m^{-3}) respectively.

(iii) *Efflux* Y → Z: follows diffusion kinetics governed by rate coefficient k_2:

$$+\frac{dz}{dt} = k_2 y \frac{V}{V_z} \tag{4}$$

As noted above, diffusive backflux $Z \rightarrow Y$ can be neglected since $z \ll y$.

From (2), (3) and (4), the rate of change of the interstitial GABA concentration y is given by:

$$\frac{dy}{dt} = k_1 x - \frac{\alpha y}{K+y} - k_2 y \qquad (5)$$

From (1):

$$x = x_0 - y - z(V_z/V) \qquad (6)$$

Substituting for x in (5):

$$\frac{dy}{dt} = k_1 x_0 - (k_1 + k_2)y - \frac{\alpha \cdot y}{K+y} - k_1 z\left(\frac{V_z}{V}\right) \qquad (7)$$

Calculation Equations (7) and (4) were integrated numerically at time intervals of 10^{-4} min using a two-stage fourth order Runge-Kutta design for x, y and dz/dt at $t>0$. From these the apparent efflux rate coefficient k_{app} was calculated as:

$$k_{app} = \frac{dz}{dt} \cdot \frac{V_z}{V} /(x+y) \qquad (8)$$

Constants The following values were selected:

(i) Initial overall intracellular GABA concentration $x_0 = 0.46$ mol m^{-3} (0.46 mM) unloaded and 1.6 mol m^{-3} (1.6 mM) loaded (i.e. after exposure to 1 mM external GABA for 60 min, see Table 3; AOAA is assumed to be present).

(ii) $V_z/V = 10^3$ (at a perfusion rate of 1 ml/min and ganglion volume 1 µl, i.e. assuming exchange at 1 min intervals, a minimum estimate).

(iii) $k_1 = 2.4 \times 10^{-3}$ min^{-1}. This is the projected maximum value for the measured efflux rate coefficient for [^{3}H]-GABA at infinite external concentrations of unlabelled GABA or inhibitor, when reaccumulation of [^{3}H]-GABA is totally inhibited. Then the middle term of equation (5) is zero, and equation (5) simplifies to

$$\frac{dy}{dt} = k_1 x - k_2 y \qquad (9)$$

If $k_1 \neq k_2$, this integrates to

$$y = \frac{k_1 x_0}{k_2 - k_1}[\exp(-k_1 t) - \exp(-k_2 t)] \qquad (10)$$

Since $k_2 \gg k_1$ (see (iv) below), at finite values of t

$$y = \frac{k_1}{k_2} \cdot x_0 [\exp(-k_1 t)] \qquad (11)$$

Combining (4) and (11) and rearranging:

$$k_1 = \frac{dz}{dt} \cdot \frac{V_z}{V} \cdot (x_0)^{-1} \qquad (12)$$

Comparing with the experimentally-measured rate coefficient in (8):

$$k_{app} = \frac{dz}{dt} \cdot \frac{V_z}{V}(x+y)^{-1} \qquad (8)$$

and with $y \ll x$, $k_{app} \approx k_1$.

(iv) The rate coefficient for efflux from the interstitial fluid $k_2 = 0.7$ min^{-1} (estimated from the desaturation curves for extracellular ^{24}Na (Brown & Scholfield, 1974) and mannitol (Brown & Scholfield, 1972)).

(v) $\alpha = 1.11 \times 10^{-2}$ mol m^{-3} min^{-1} (the experimentally-determined maximal velocity for the carrier-mediated accumulation of [^{3}H]-GABA: Bowery, 1974). (K was varied, see Results below).

Results of calculations

Figure 11 shows how the calculated interstitial GABA concentration (y) and the rate coefficient for efflux of GABA from the tissue (k_{app}) change with time. The

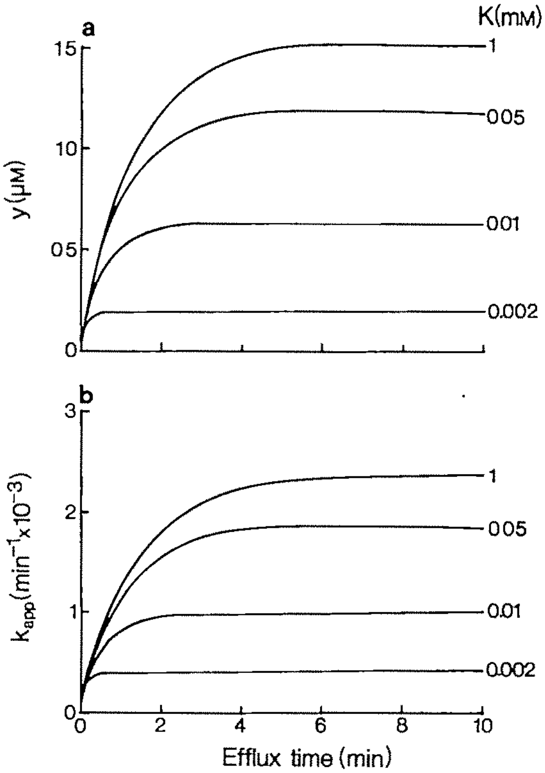

Figure 11 Calculated interstitial γ-aminobutyric acid (GABA) concentration (a) and tissue efflux rate coefficient (b) at $x_0 = 0.46$ mM (unloaded ganglion) and $K = 2$ µM – 1 mM, plotted against time.

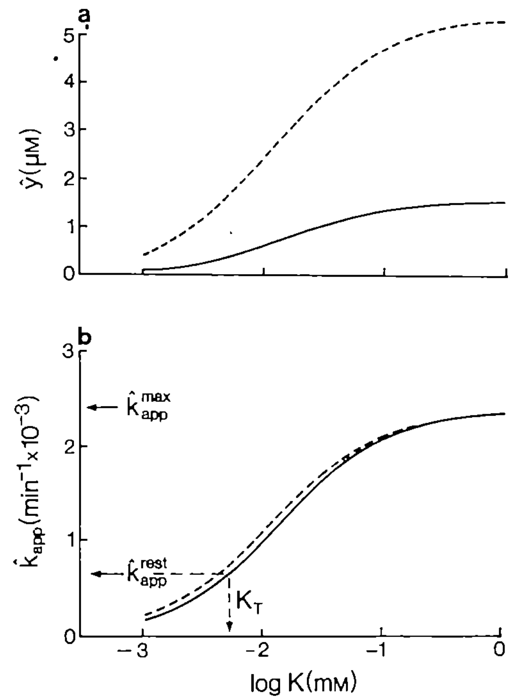

Figure 12 Calculated maximum interstitial GABA concentration (a) and tissue efflux rate coefficients (b) plotted against K (on a logarithmic scale) for unloaded ganglia (solid line, x_0=0.46 mM) and for ganglia previously loaded with GABA (x_0=1.6 mM). Arrows indicate values appropriate to the experimentally-observed resting efflux rate coefficient (0.64×10^{-3} min^{-1} at x_0=0.46 mM).

ganglion was assumed not to have been previously loaded with GABA (x_0=0.46 mM) and K was varied. The maximum value of k_{app} ($\hat{k}_{app}$), although delayed compared with $\hat{y}$, was attained by 4 min except at very high values for K (i.e. corresponding to very high external substrate concentrations, see below): hence the efflux rate coefficient measured after 4 min (see Figure 3) provides a good approximation to $\hat{k}_{app}$.

In Figure 12 values for $\hat{y}$ and $\hat{k}_{app}$ are plotted against K on a logarithmic scale for the two conditions where the ganglion has or has not been previously incubated in GABA (x_0= 1.6 and 0.46 mM respectively). Considering first the *rate coefficient*, the following points emerge:

(i) The absolute value of $\hat{k}_{app}$ shows a hyperbolic dependence upon K such that

$$\hat{k}_{app} = \frac{\hat{k}_{app}^{max} \cdot K}{\phi + K} \qquad (13)$$

where ϕ is the value of K corresponding to $\hat{k}_{app} = 0.5\,\hat{k}_{app}^{max}$. This latter value ($2.4 \times 10^{-3}$ min^{-1}) is numerically equivalent to k_1, as predicted in equations (8) and (12).

(ii) At the mean resting rate coefficient measured experimentally for [^{3}H]-GABA efflux in the absence of external substrate (0.64×10^{-3} min^{-1} at x_0= 0.46 mM), K= 5.4 μM. This should correspond to K_T, the Michaelis constant for GABA as a substrate for the inward carrier. In fact it is slightly lower than the values previously measured for the high-affinity influx process (7–8 μM: Bowery & Brown, 1972; Bowery, 1974) or that derived from the overall IC$_{50}$ (14.3 μM, Figure 1). This discrepancy is probably acceptable if the following points are borne in mind: (a) k_{app}^{rest} measured experimentally showed great variation. Further, the value of 0.64×10^{-3} refers to that attained after a rather prolonged period of efflux, which, at low values of K, would tend to under-read the peak value. The latter would be attained after only a few minutes efflux (see Figure 11), but would not be open to experimental measurement because of interference by clearance of extracellular radioactivity. (b) The position of the curve also depends critically upon the maximal uptake velocity, which is subject to further measurement uncertainty.

(iii) The effect of adding an external substrate S is to increase K above K_T such that

$$K = K_T(1 + S/K_S) \qquad (14)$$

where S is the external substrate concentration and K_s is the concentration of S producing half-saturation of the carrier. (Clearly, if the efflux of radioactive GABA is measured and S is unlabelled GABA, $K_S = K_T$). Thus, when the fractional *increase* in the efflux rate coefficient for labelled GABA is plotted against the external unlabelled substrate concentration a hyperbolic curve results (Figure 13) as previously obtained experimentally (cf. Figure 3). However, it should be noted that the concentration of substrate producing a half-maximal acceleration of efflux (designated earlier as K_E) is *not* equal to K_S but (in this case) equals $4K_S$. This numerical relationship between K_E and K_S is not invariate but depends upon the value of K_T, the Michaelis constant for GABA-uptake. Thus, in the presence of a re-uptake system, the absolute value of K_S cannot be determined directly from the efflux acceleration unless K_T is known, though measurements of relative affinities for the inward carrier can, of course, be made.

(iv) The effect of increasing x_0 to 1.6 mM is to shift the curve in Figure 12 to the left (ϕ in equation (13) reduced from 15.3 to 13.3 μM) and hence to raise the resting efflux rate coefficient. This results from the increased interstitial GABA concentration (Figure 12a): in consequence a smaller proportion of effluent GABA is re-accumulated, leading to a greater overflow. At K_T = 5.4 μM, the resting rate coefficient is increased from 0.64 to 0.75×10^{-3} min^{-1}, but the maximum rate coefficient at high external substrate

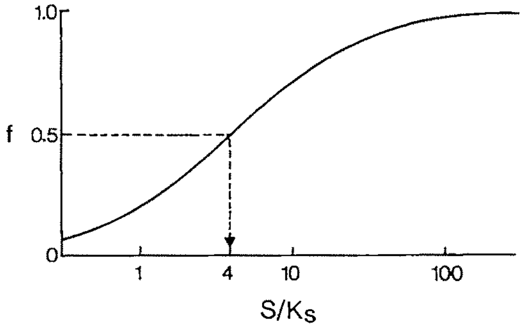

Figure 13 Transposition of data in Figure 12b to the form of Figure 3, in which the fractional increase in efflux rate coefficient $f = \Delta\hat{k}/\Delta\hat{k}^{max}$ is plotted against the external substrate concentration in units S/K_S, where

$$\Delta\hat{k} = (\hat{k}_{app}/\hat{k}^{rest}_{app}) - 1$$

and

$$\Delta\hat{k}^{max} = (\hat{k}^{max}_{app}/\hat{k}^{rest}_{app}) - 1$$

$\hat{k}^{rest}_{app}$ was taken as 0.64×10^{-3} min^{-1} and $\hat{k}^{max}_{app}$ as 2.4×10^{-3} min^{-1}. S/K_S was calculated from equation (14) assuming K_T = 5.4 μM [GABA]. The curve is hyperbolic such that

$$f = \frac{S/K_S}{4 + S/K_S}$$

concentration is unchanged. This accords with the trend of the experimental observations (see Results section).

Interstitial GABA concentrations Some values for *y* abstracted from Figure 12a are listed in Table 4. The following points seem worth emphasizing.

(i) Even in the absence of external substrate, at $K_T = 5.4$ μM maximal interstitial GABA concentrations of 0.41 and 1.64 μM may be anticipated to result from the exit of GABA from normal and preloaded tissue respectively. The latter value is not negligible in view of the neuronal sensitivity, and may account for the slight modification on the dose-response curves for GABA and 3-APS after loading.

(ii) A 4 min exposure to 300 μM BALA or 3 mM BABA might increase this interstitial concentration by some 0.9 μM in unloaded ganglia and 2.8–2.9 μM in loaded ganglia. Hence the increase in the interstitial GABA concentration produced by these agents consequent upon loading would be 1.9–2.0 μM, rising to a limit of 2.5 μM at very high concentrations. These values correspond very well with those calculated from the shift in the experimental dose-response curves after loading.

(iii) The above figures also suggest that, at low concentrations, the depolarizing actions of BABA and BALA can be accounted for entirely by the increased interstitial GABA concentration, and not by direct receptor-activation. Thus, the response to 3 mM BABA in unloaded ganglia corresponded to the action of 1 μM GABA, which accords with the increment in interstitial GABA (0.9 μM) very precisely.

Limitations

The model is simplified in that it does not take into account the anatomical distribution of GABA in the ganglion or possible gradients in the interstitial GABA concentration. Neither limitation may be too serious.

Table 4 Calculated interstitial γ-aminobutyric acid (GABA) concentrations from Figure 12

	Condition	*Interstitial [GABA] (μM)* *a* *Unloaded* $x_0 = 0.46$ mM	 *b* *Loaded* $x_0 = 1.6$ mM	 *c* *b−a*
(1)*	Rest	0.41	1.64	1.23
(2)†	300 μM BALA	1.32	4.42	3.10
(3)	(2)−(1)	0.91	2.78	1.87
(4)†	3 mM BABA	1.30	4.54	3.24
(5)	(4)−(1)	0.89	2.90	2.01
(6)	At $S = \infty$, $t = 4$ min	1.45	5.05	3.60
(7)	(6)−(1)	1.04	3.41	2.37
(8)	At $S = \infty$, $t = \infty$	1.55	5.32	3.77
(9)	(8)−(1)	1.14	3.68	2.54

* Assuming K_T = 5.4 μM. † After 4 min application.

(i) The GABA is not evenly distributed in the intracellular space but is (probably) confined entirely to glial cells. This would not affect calculations of average interstitial concentrations since the smaller intracellular volume will be offset by a higher concentration than that assumed on the basis of an even intracellular distribution.

(ii) It might be anticipated that higher concentrations would arise (transiently) in the narrow clefts between satellite cells and neurones than elsewhere in the interstitial space. However, this would be offset by a faster redistribution into the remaining interstitial space than that for total extracellular clearance. Further, the Schwann cells surrounding fibres may be a quantitatively much larger source of GABA than the satellite cells, since the uptake of GABA by sympathetic nerve trunks exceeds that of the ganglion itself (Bowery & Brown, 1972).

Alternative models

The congruence between predictions and observations suggests that the above concept of inhibited re-accumulation is appropriate for this system. However, it may be noted that the same approach would also encompass accelerated exchange diffusion, with certain restrictions. These would be that either x_0 is invariate and x does not change appreciably with time, or that the internal face of the outward carrier is very unsaturated: in both cases the efflux $X \rightarrow Y$ would be regarded as governed by the single rate coefficient k_1. However, the effect of varying x_0 appears incompatible with such a process in the ganglion. Thus, one explanation for accelerated exchange diffusion might be that the outward transport rate is normally submaximal because the internal substrate concentration is too low to saturate the inward face of the carrier. If no re-accumulation occurs the degree of saturation may be calculated from the ratio of the resting and maximal driven efflux rate coefficients at any given internal GABA concentration. In the ganglion at $x_0 = 0.46$ mM, this ratio is 0.27, giving a value for K_x of 1.24 mM. If now x_0 is raised to 1.6 mM, the efflux rate coefficient will fall from 0.64 to 0.39×10^{-3} min^{-1}; further, the maximum rate coefficient attained at high external substrate concentration will be only 0.69×10^{-3} min^{-1}, instead of 2.4×10^{-3} min^{-1}, since the inward-facing carrier sites show a greater degree of saturation (0.56 as against 0.27 at $x_0 = 0.46$ mM). Both effects are contrary to our experimental observations. An alternative view is that the internal face of the carrier is fully saturated but outward mobility is low, perhaps because of the low internal Na$^+$ concentration (see Martin, 1973). In this case, however, both resting and stimulated efflux rate coefficients should fall in proportion to the increase in x_0. These objections need not necessarily hold in other tissues, of course, and Levi & Raiteri (1974) and Raiteri *et al.* (1975) have recently suggested that accelerated exchange diffusion may be responsible for the substrate-stimulated efflux of GABA from synaptosomes.

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THE INHIBITION BY GLUCAGON OF THE VASOCONSTRICTOR ACTIONS OF NORADRENALINE, ANGIOTENSIN AND VASOPRESSIN ON THE HEPATIC ARTERIAL VASCULAR BED OF THE DOG

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1 The hepatic artery of the anaesthetized dog was cannulated and perfused from a femoral artery, the blood flow and perfusion pressure being monitored continuously. The sympathetic periarterial nerves were divided.

2 Dose-dependent increases in hepatic arterial vascular resistance (HAVR) resulted from intra-arterial injections of noradrenaline, angiotensin and vasopressin.

3 Single injections of glucagon (100 μg, i.a.) caused a transient significant fall in HAVR of 19.9 ± 3.2%, and infusions of 25 μg/min of glucagon intra-arterially caused maintained reductions in HAVR of 16.9 ± 4.2%.

4 After single injections of 100 μg glucagon intra-arterially the vasoconstrictor responses to noradrenaline, angiotensin, and vasopressin were reduced by about 85–95%. Recovery occurred in 8–10 minutes.

5 Intra-arterial infusions of glucagon, 2.5–50.0 μg/min, reduced the effects of test doses of noradrenaline, angiotensin and vasopressin throughout the period of the infusions.

6 Dose-response curves to the constrictor agents were constructed before, during and after intra-arterial infusions of 25 μg/min of glucagon. Glucagon caused a parallel shift of the curves for noradrenaline and angiotensin to the right, with no suppression of the maximum response.

7 Infusions of glucagon shifted the dose-response curve for vasopressin to the right, but, in contrast to noradrenaline and angiotensin, the shift was nonparallel and there was a suppression of the maximum response by about one-half.

8 A large dose of insulin, 10 iu, transiently reduced HAVR and caused a weak and very transient inhibition of the effect of test doses of noradrenaline. The characteristics of these effects were quite different from those of glucagon.

9 It is possible that the antagonism by glucagon of the vasoconstrictor responses of the hepatic arterial vasculature may be important in protecting this vascular bed from the effects of concomitantly released vasoconstrictor agents.

Introduction

Pancreatic glucagon is secreted during hypoglycaemia to break down hepatic glycogen and mobilize glucose. It has weak vasodilator properties when introduced into the dog's liver by either hepatic arterial (Bashour, Geumei, Nafrawi & Downey, 1973) or portal venous (Kock, Roding, Hahnloser, Tibblin & Schenk, 1970) routes. In addition, it is reported to antagonize the vasoconstrictor actions of adrenaline, noradrenaline and sympathetic nerve stimulation on the superior mesenteric arterial vascular bed (Kock, Tibblin & Schenk, 1971).

The present experiments were performed to investigate the extent and nature of the antagonism of noradrenaline by glucagon on the hepatic arterial bed in the dog, and in addition to examine whether this antagonism was specific to catecholamines by studying the vasoconstrictor properties of the polypeptides, angiotensin and vasopressin, in the hepatic arterial vasculature and subsequently determining the extent and characteristics of any inhibition by intra-arterial administration of glucagon.

A preliminary report of part of the results of this investigation has been published previously (Richardson & Withrington, 1975).

Methods

Twelve dogs (13.4 ± 2.9 kg: mean ± s.d., range 10.4–19.0 kg) were starved for 24 h before induction of anaesthesia with 7.5–10.0 mg/kg methohexitone sodium (Brietal, Lilly), intravenously. Anaesthesia was maintained with chloralose (Kuhlmann, Paris: 50 mg/kg) and urethane (BDH; 500 mg/kg) intravenous supplements being given when necessary to maintain a constant level of anaesthesia.

After a midline laparotomy, the hepatic artery was separated from its periarterial postganglionic nerves which were divided. Blood coagulation was prevented with intravenous heparin 200 iu/kg (Weddel Pharmaceuticals) followed by hourly supplements of 100 iu/kg. The left femoral artery was cannulated, and blood diverted from this artery to perfuse the hepatic arterial vascular bed through the cannulated common hepatic artery; the blood flow through this cannula to the hepatic artery (hepatic arterial blood flow; HABF) was measured with a cannulated flowhead and electromagnetic flowmeter (CardioVascular Instruments Ltd.), occlusive flow zeros being established regularly throughout the course of each experiment. Hepatic arterial perfusion pressure (PP) was measured from a 'T' piece in the cannula close to the point of cannulation of the hepatic artery, with a Bell & Howell L220 strain gauge transducer. To minimize any pressure drop in the cannula system, wide-bore tubing (5.0 mm i.d.) was used, and the length of the tubing kept to a minimum. The cannula system carried additional 'T' pieces for the intra-arterial injection and infusion of vasoactive agents.

To ensure that the blood flow measured was the arterial inflow to the liver only, the extrahepatic branches of the common hepatic artery (gastroduodenal and right gastric arteries) were occluded. The abdominal incision was then closed, a thermometer inserted into the abdomen, and the intra-abdominal temperature maintained at 37–38°C. Drug administration was started after stable control variables had been recorded for 20 minutes.

To monitor possible systemic effects of vasoactive agents administered intra-arterially to the liver, phasic systemic arterial pressure was measured with a Statham P23Gb strain gauge transducer from a cannulated common carotid artery, and mean systemic arterial pressure and heart rate were derived electronically from this measurement. After appropriate amplification, all variables were displayed on a Devices M-19 recorder.

Calculation of results

Hepatic arterial mean perfusion pressure (PP) was calculated as diastolic plus one-third of the pulse pressure; this calculated mean pressure was in agreement with the value obtained by switching the phasic output through an averaging circuit with a time constant of about 3 seconds.

Hepatic arterial mean bloodflow (HABF) was derived electronically by passing the phasic waveform through an averaging circuit with a time constant of 0.6 s; mean and phasic blood flows were displayed simultaneously.

Hepatic arterial vascular resistance (HAVR) was calculated as hepatic arterial mean perfusion pressure (mmHg) divided by hepatic arterial mean blood flow (ml min^{-1}, or ml min^{-1} 100 g^{-1}) and expressed in mmHg ml^{-1} min, or mmHg ml^{-1} min 100 g.

Changes in vascular resistance were calculated as percentage changes from control values immediately prior to any procedure, i.e. (peak vascular resistance – control vascular resistance) × 100/ control vascular resistance.

Liver weight was obtained immediately after each experiment. Values for blood flow and vascular resistance expressed per 100 g refer to this terminal weight of liver.

Expression of results

Except where indicated to the contrary, values are expressed as means ± s.e. means. The significance of differences between paired data was assessed using Student's *t* test.

Drugs

The drugs used were: angiotensin amide (Hypertensin, Ciba), glucagon hydrochloride (Lilly), neutral insulin (Nuso, Wellcome), noradrenaline acid tartrate (Levophed, Winthrop) and vasopressin (Pitressin for i.v. injection, Parke-Davis). Doses of angiotensin are expressed in terms of the salt, of noradrenaline and glucagon in terms of the bases, and of insulin and vasopressin in international units of activity. For vasopressin, 1 unit is equivalent to 0.5 mg (manufacturer's data).

Drugs were dissolved in, or diluted with 0.9% w/v NaCl solution (saline). Intra-arterial injections were made at a point between the flow probe and the hepatic arterial cannula in volumes not exceeding 0.5 ml, washed in with saline to a total injectate volume of 1.5 ml. This resulted in a constant injection artifact visible on both the flow and pressure records (Figures 2 and 4) and clearly distinguishable from the subsequent drug-induced changes in hepatic arterial blood flow.

Intra-arterial infusions were at a rate of 1.0 ml/min from a precalibrated roller pump (Watson-Marlow MHRE-200).

The volume of the external circuit was compensated for with a solution of low molecular weight dextran in normal saline (Rheomacrodex, Pharmacia).

Results

Control values

The livers weighed 331.7 ± 75.8 (s.d.) g, representing 2.53 ± 0.56 (s.d.) % of the weight of the dogs. Under control conditions, the hepatic arterial blood flow was 45.9 ± 4.5 ml min^{-1} 100 g^{-1}, being positively and significantly correlated with the liver weights ($r = +0.299$; $P < 0.05$). The hepatic arterial perfusion pressure was 121.6 ± 6.6 mmHg, and the calculated hepatic arterial vascular resistance, 2.88 ± 0.28 mmHg ml^{-1} min 100 g, or 0.90 ± 0.10 mmHg ml^{-1} min ($n = 11$).

The effects of noradrenaline, angiotensin and vasopressin on the hepatic arterial vascular resistance

Noradrenaline, angiotensin and vasopressin were injected into the hepatic artery in increasing doses to establish the dose-response relationship. The results from 9 experiments are summarized in Figure 1, where the increase in hepatic arterial vascular resistance (HAVR) is expressed as a percentage of the maximum increase attained in individual experiments. The changes in perfusion pressure, hepatic arterial blood flow and calculated hepatic arterial vascular resistance at maximum vasoconstriction due to noradrenaline, angiotensin and vasopressin are shown in Table 1.

Although the maximum rises in HAVR appear to be different for the three vasoconstrictors, small differences in low blood flows attained at maximal vasoconstriction may give rise to very large differences in calculated vascular resistance. In experiments where complete dose-response curves were constructed for two different vasoconstrictors, paired analysis revealed no significant differences between the minimum hepatic arterial blood flows attained at maximal vasoconstriction ($P > 0.10$). On the basis of these experiments alone, it is not therefore justifiable to rank the three vasoconstrictor agents in any order of maximum vasoconstrictor potency.

The increases in calculated HAVR, when large doses of the vasoconstrictor agents were injected, arose from very substantial reductions in hepatic arterial blood flow which were, however, inevitably accompanied by small rises in perfusion pressure (PP). A rise in PP itself leads to an increase in HAVR due to a myogenic reaction (Bayliss, 1902; Folkow, 1964; Mellander & Johansson, 1968). A correction needs to be applied to determine whether this contribution to the changes in HAVR is significant. The pressure-flow curve for the canine hepatic arterial vascular bed has been established in similar preparations (Torrance, 1961) and calculations from these data reveal that, in the present experiments, the proportion of the total calculated rises in HAVR due to noradrenaline which is attributable to the myogenic response to increases in PP is 3.0 ± 1.0%. The corresponding proportions for

Table 1 The effects of doses of noradrenaline, angiotensin and vasopressin which in each experiment produced maximum vasoconstriction, upon hepatic arterial perfusion pressure, blood flow, and calculated vascular resistance

	PP (*mmHg*)	*PP*	*HABF* (*ml min^{-1} 100 g^{-1}*)	*HABF*	*HAVR* (*mmHg ml^{-1} min 100 g*)	*HAVR*
	Control	*Max*	*Control*	*Max*	*Control*	*Max*
a. Noradrenaline						
	104.8 ± 9.0	125.5 ± 9.7	36.9 ±8.5	6.9 ±3.1	4.09 ±1.28	66.74 ±41.10
b. Angiotensin						
	110.2 ± 4.9	135.6 ± 4.1	61.7 ±7.9	29.4 ±6.6	1.88 ±0.20	5.26 ±0.79
c. Vasopressin						
	113.0 ± 8.4	134.5 ± 7.3	53.4 ±7.4	19.5 ±5.2	2.38 ±0.40	7.53 ±1.25

All values are means ± s.e. means for values immediately before (Control) and at the peak of maximum vasoconstriction (Max) for mean perfusion pressure (PP), mean hepatic arterial blood flow (HABF) and calculated vascular resistance (HAVR). For noradrenaline and vasopressin, $n = 6$, and for angiotensin, $n = 5$.

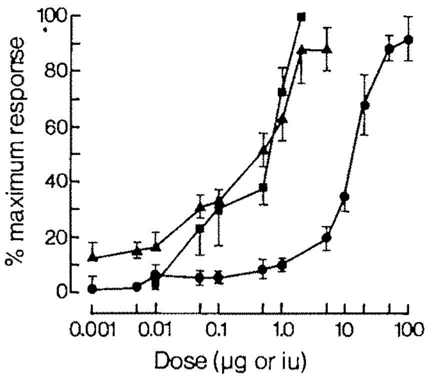

Figure 1 Dose-response curves for increase in hepatic arterial vascular resistance to intra-arterially administered noradrenaline (●), angiotensin (▲), and vasopressin (■). Abscissa scale: $\log_{10}$ dose of drug in µg (noradrenaline, angiotensin) or international units (vasopressin). Ordinate scale: percentage of the maximum increase in hepatic arterial vascular resistance found in each experiment. Points represent the means of 6 experiments (5 for angiotensin). Vertical bars show the s.e. means.

angiotensin and vasopressin were 9.0±0.6% and 6.2±1.9% respectively. These small effects have no influence on the form or position of the curves, expressed as percentages of the maxima (Figure 1).

The effect of intra-arterial glucagon on the hepatic arterial vascular bed

Injection. Glucagon was injected in a dose of 100 µg base intra-arterially on 14 occasions to 5 preparations, resulting in a transient and significant ($P<0.001$) reduction in hepatic arterial vascular resistance of 19.9±3.2% (Figure 2). The hepatic arterial vascular resistance returned to the control values 183±36 s after the injection of glucagon.

Infusion. Glucagon was infused at a rate of 25 µg/min intra-arterially on 6 occasions in 5 preparations, resulting in a significant ($P<0.02$) reduction in HAVR of 16.9±4.2% (Figure 4). This reduction in HAVR persisted throughout the period of the infusion of glucagon.

Effects of single intra-arterial injections of glucagon on the responses of the hepatic arterial bed to noradrenaline, angiotensin and vasopressin

Single doses of noradrenaline (10 µg) angiotensin (0.5 µg) and vasopressin (0.1 or 0.2 iu) were chosen that lay on the linear part of the dose-response curve and produced about 50% maximal vasoconstriction (Figure 1). The test injections were made before, and at set intervals after single intra-arterial injections of glucagon (100 µg) to allow the time-course of both the onset and the recovery of any changes in the hepatic vasoconstrictor test responses to be clearly ascertained.

Noradrenaline. The test dose of noradrenaline (10 µg) was injected into the hepatic artery on 52 occasions in 9 preparations, resulting in a rise in HAVR of 229.4±27.5%. One minute after the single arterial dose of glucagon (100 µg), this hepatic arterial vasoconstrictor response to noradrenaline was reduced to 14.9±4.2% of the control ($n=6$). The vasoconstrictor response to noradrenaline returned to the control level 8–10 min after the injection of glucagon (Figure 3).

Angiotensin. The test dose of angiotensin (0.5 µg) was injected intra-arterially on 27 occasions to 7 preparations, resulting in an increase in HAVR of 118.7±13.4%. One minute after the injection of glucagon, the increase in HAVR in response to the test dose of angiotensin was reduced to 5.1±2.4% of the control in 4 tests. The vasoconstrictor response to the test dose of angiotensin returned to control within 8–10 min (Figure 3).

Vasopressin. The test dose of vasopressin which was used varied between experiments according to the sensitivity of the preparations; the test dose was either 0.1 or 0.2 iu, the dose being that which caused approximately 50% of the maximum vasoconstriction. The test dose of vasopressin was administered on 26 occasions in 7 preparations and provoked an increase in HAVR of 66.4±9.2%. Within 1 min of the glucagon (100 µg) injection, the response to vasopressin was reduced (Figure 2), and in 4 tests, the mean reduction was to 11.0±4.5% of the control. The time course of the recovery was similar to the other test vasoconstrictors (Figure 3).

In summary the hepatic arterial vasoconstrictor responses to all three substances, noradrenaline, angiotensin, and vasopressin, were significantly but transiently reduced by single intra-arterial injections of glucagon.

Effects of hepatic arterial infusions of glucagon on the hepatic vasoconstrictor responses to noradrenaline, angiotensin and vasopressin

To determine whether the reduction of the vasoconstrictor responses to noradrenaline, angiotensin and vasopressin was transient, even in the continued presence of glucagon, the test doses of each vasoconstrictor were injected into the hepatic arterial circulation during a maintained infusion of glucagon (2.5 to 50.0 µg/min) into the hepatic artery.

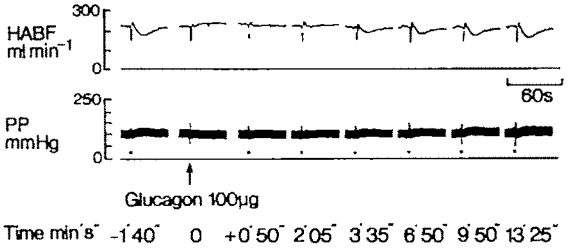

Figure 2 The effects of intra-arterial injections of 0.1 unit of vasopressin (at dots) on hepatic arterial blood flow (HABF) and perfusion pressure (PP) before and at intervals after the intra-arterial injection of 100 µg glucagon. The times of the injections of vasopressin relative to the injection of glucagon are shown (min' s") below the records.

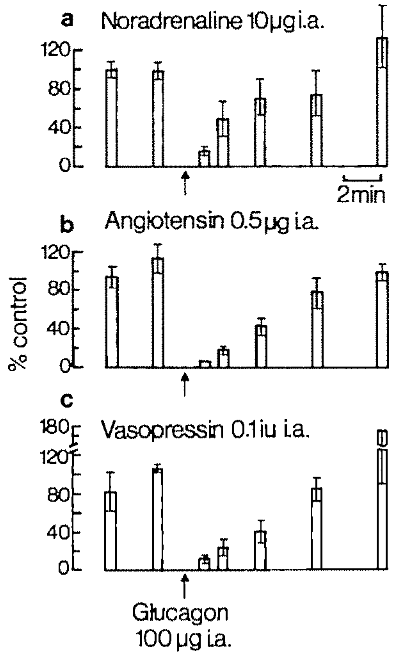

Figure 3 The effects of single injections of 100 µg of glucagon intra-arterially upon the responses of the hepatic arterial vasculature to (a) noradrenaline (10 µg i.a. injections), (b) angiotensin (0.5 µg i.a. injections) and (c) vasopressin (0.1 iu i.a. injections). Abscissa scales: time in min; ordinate scales: percentage of the initial control increase in HAVR produced by the test doses of each drug. Columns represent the means from 4 preparations and the vertical lines show the s.e. means.

Suppression of the hepatic vasoconstrictor responses to the test doses was observed (Figure 4) and persisted throughout the infusion periods of 12–15 minutes. There was no evidence of the antagonist action of glucagon to any of the vasoconstrictors either increasing or decreasing during the course of the infusions. When the infusion of glucagon was stopped, the responses to the test doses returned to control values within 3 min, although in some experiments the recovery of the vasoconstrictor effects of vasopressin was slightly slower.

In any single experiment, the suppression of the vasoconstrictor responses was related to the dose of glucagon infused (from 2.5 to 50.0 µg/min) and therefore to the resultant hepatic arterial blood concentration. However, the dose of glucagon required to induce suppression of vasoconstrictor responses varied between experiments, and without considerable further experimentation, no quantitative analysis can be made to relate blood concentration of glucagon to the extent of suppression of the responses to each of the vasoconstrictor test substances. This variation in the dose of glucagon required for suppression may be related to variations in the hepatic arterial blood distribution, and in the transport and fate of glucagon in the blood and within the liver.

The nature of the antagonism between glucagon and noradrenaline, angiotensin, and vasopressin in the hepatic arterial vascular bed

The magnitude of the antagonism of the hepatic vasoconstrictor responses to the test substances by intra-arterial glucagon whether injected (Figure 3) or infused (Figure 4) was very similar. In addition, the time-course of the onset and recovery from the antagonism was qualitatively the same for each vasoconstrictor.

To determine whether the nature of the antagonism

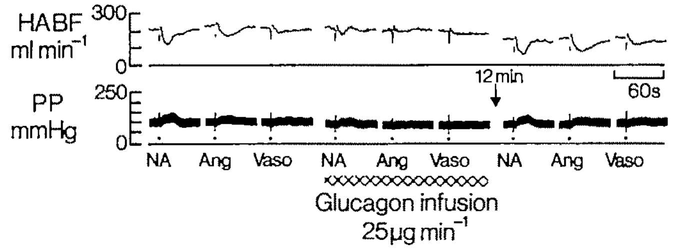

Figure 4 Effect of an intra-arterial infusion of glucagon 25 µg min^{-1} on the change in hepatic arterial blood flow (HABF) and perfusion pressure (PP) brought about by intra-arterial injections of noradrenaline (NA 10 µg), angiotensin (Ang 0.5 µg), and vasopressin (Vaso 0.1 iu). The period of the infusion is shown by the horizontal hatched bar; during this period the changes due to all three constrictor agents are attenuated compared with the control responses before and after the infusion.

was the same for noradrenaline, angiotensin and vasopressin, the characteristics of the interaction between intra-arterial glucagon and each of the vasoconstrictors was assessed. Complete dose-response curves to each substance were determined by increasing intra-arterial injections before, during, and after the intra-arterial infusion of glucagon (25 µg/minute).

Noradrenaline. In 3 experiments, graded doses of noradrenaline (0.01 to 50 µg) were injected into the hepatic artery before, and after the glucagon infusion to establish the control relationship between dose and change in HAVR. There was no significant alteration in the responses due to the interposed infusion of glucagon. The infusion of glucagon caused a parallel and marked shift of the noradrenaline dose-response curve to the right (Figure 5a) but increasing the dose of noradrenaline revealed that the maximum increase in HAVR due to noradrenaline during the infusion of glucagon was not suppressed although there was a considerable increase in the dose of noradrenaline required to evoke this maximum response (Table 2).

Angiotensin. An identical procedure to that used for noradrenaline was adopted to examine the antagonism of glucagon to angiotensin. The result of one experiment (Figure 5b) clearly showed a parallel shift of the dose-response curve to the right with no suppression of the maximum increase in HAVR, although the dose at which the maximum increase in HAVR was attained was higher than that in the absence of glucagon (Table 2).

Vasopressin. Graded doses of vasopressin were injected into the hepatic artery to establish the dose-

Table 2 Hepatic arterial vascular resistance increases resulting from the intra-arterial injection of doses of noradrenaline, angiotensin, and vasopressin causing maximal vasoconstriction before and during the infusion of 25 µg/min glucagon

	Dose causing maximal vasoconstriction	*HAVR control (mmHg ml^{-1} min)*	*HAVR maximum (mmHg ml^{-1} min)*
a. Noradrenaline			
Control	20 µg	0.82	19.20
During glucagon	50 µg	0.66	14.89
b. Angiotensin			
Control	2 µg	0.60	1.68
During glucagon	5 µg	0.61	1.72
c. Vasopressin			
Control	1 u	0.63	1.80
During glucagon	2 u	0.54	0.87

These values are from the same experiments as are illustrated in Figure 5a, 5b and 5c.

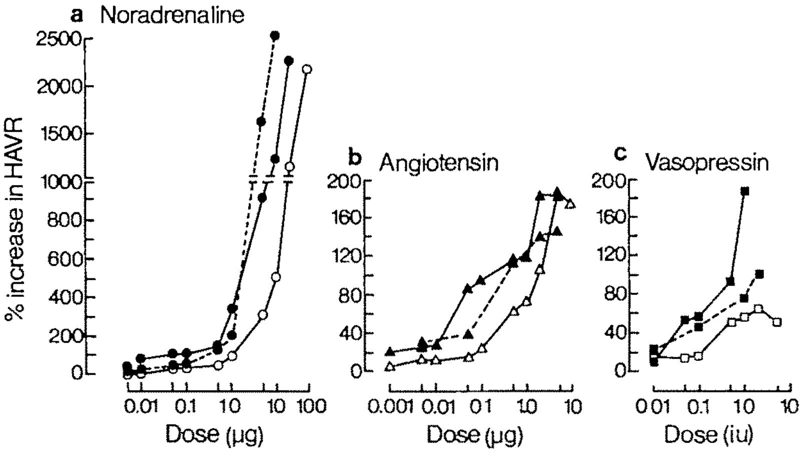

Figure 5 Dose-response curves obtained in different experiments to (a) noradrenaline, (b) angiotensin and (c) vasopressin. In each case the abscissa scale is $\log_{10}$ dose and the ordinate scale the absolute percentage increase in HAVR. The solid symbols show the dose-response curves before (solid lines) and after (broken lines) the i.a. infusion of 25 µg min^{-1} glucagon, and the open symbols show the corresponding dose-response curves during the glucagon infusions.

response curve. The infusion of glucagon (25 µg/min) produced a non-parallel shift of the dose-response curve to the right of the control dose-response curves determined both before and after the glucagon infusion (Figure 5c). In addition, the intra-arterial infusion of glucagon caused a substantial suppression of the maximum increase in HAVR achieved in response to arterial vasopressin in each of three experiments. During the infusion of glucagon, the maximum increase in HAVR attained with vasopressin was 45.4 ± 15.3% of the control maxima (Table 2).

These observations suggest a fundamental difference in the nature of the antagonism between glucagon and vasopressin, and that between glucagon and both noradrenaline and angiotensin.

Insulin

Insulin was injected intra-arterially to the liver in the large dose of 10 iu on 5 occasions to 4 preparations. This caused a variable and insignificant ($P > 0.05$) rise in hepatic arterial vascular resistance of 32.9 ± 13.5%. One minute after these injections of insulin, the vasoconstrictor effects of test doses of noradrenaline (10 µg, i.a.) were reduced to 69.1 ± 7.8% of the effects before the injections of insulin ($P < 0.05$) but the effect of noradrenaline had returned to 98.9 ± 11.8% of the initial control effect 2 min after the insulin injections, in contrast to the more marked and persistent attenuation of the effects of the vasoconstrictors by glucagon.

Discussion

The control values for hepatic arterial blood flow, hepatic arterial vascular resistance and liver weight reported in these experiments accord well with previously published data from the same species (Green, Hall, Sexton & Deal, 1959; Torrance, 1961). At the end of 3 to 4 h of perfusion, the livers were of good colour and showed no obvious signs of swelling or the 'blueness' reported, after more extensive operative procedures, by other investigators (Andrews, Hecker, Maegraith & Ritchie, 1955).

The vasoconstrictor properties of single doses of noradrenaline on the hepatic arterial vascular bed have been established for some time (Grayson & Johnson, 1953; Andrews *et al.*, 1955; Green *et al.*, 1959), and the results presented in this paper establish the complete dose-response relationship. Vasoconstriction was the only effect seen, with no evidence of vasodilatation in any experiment, at even the lowest doses. The maximum increase in HAVR attained with the largest intra-arterial doses of noradrenaline showed a very wide range between experiments (from 270 to 2250%), indicating a considerable variation of sensitivity to the natural transmitter. The highest increases in HAVR represented almost complete cessation of hepatic arterial blood flow (Table 1). Reasons for the wide variation in the absolute maximum response may include variations in uptake processes associated with the sympathetic nerve terminals, variations in degradative enzyme activity within the liver, and structural variations in the hepatic arterial vascular bed; all of these factors may depend on the age and sex of the animals, which varied.

The effects of other naturally-occurring vasoactive agents on the hepatic vascular bed has been less well established. The present experiments establish the complete dose-response curve to arterial administration of angiotensin; single large (10 µg i.a.) injections have previously been shown to cause vasoconstriction in the bovine liver (Kelly & Nyhus, 1966). In the present experiments vasoconstriction was observed at all doses, and there was no sign of tachyphylaxis to repeated injections. Angiotensin tachyphylaxis varies between species (Khairallah, Page, Bumpus & Turker, 1966) and between vascular beds in the same species (Jonsson, Svanvik & Vikgren, 1967), and may be related to the presence or absence of degradative enzymes particularly angiotensinase-A (Khairallah *et al.*, 1966). The variation in the maximum increase in HAVR in response to angiotensin was not great in the present experiments (109–280%).

Previous reports of the effects of vasopressin on the hepatic arterial vascular bed have suggested a small and variable vasoconstriction (Heimberger, Teramoto & Schumacker, 1960; Greenway & Stark, 1971); the present experiments have established the complete dose-response curve for vasopressin, and hepatic arterial vasoconstriction was the only effect observed. The maximum rises in HAVR in different experiments were between 145 and 630%, values intermediate between the maxima for noradrenaline and angiotensin.

Despite the variations in the maximum increases in HAVR found with the three vasoconstrictors, in experiments where two complete dose-response curves were completed, paired analysis revealed no significant differences between the minimum hepatic arterial blood flows at maximum vasoconstriction. The present experiments do not therefore constitute evidence for ranking the three vasoconstrictor agents in any order of maximum vasoconstrictor potency on the basis of the observed increases in hepatic arterial vascular resistance.

Glucagon caused a reduction in hepatic arterial vascular resistance both when injected and when infused into the hepatic artery, an effect similar in character and magnitude to that observed by Bashour *et al.* (1973) and which has been shown to be present in only certain vascular beds (Kock, Tibblin & Schenk, 1970). In the superior mesenteric arterial vascular bed, the vasodilatation due to glucagon has been shown not to be the result of either α-adrenoceptor blockade, or β-adrenoceptor stimulation (Tibblin, Kock & Schenk, 1970).

The present experiments confirm in principle, and extend the observations that glucagon attenuates the effects of α-adrenoceptor stimulation by adrenaline, noradrenaline or sympathetic nerve stimulation. These observations were made in the superior mesenteric arterial bed of the dog (Kock *et al.*, 1971) where, in addition, the reflex vasoconstrictor effects of haemorrhage were reduced by glucagon.

In the present paper the observations have been deliberately confined to the hepatic arterial bed, where the antagonism of noradrenaline by arterial injections of glucagon has been shown to be transient yet highly significant, and furthermore, by determination of the complete dose response curves before, during and after glucagon, the antagonism has been shown to be of the competitive variety, exhibiting the features of a parallel shift of the dose-response curve to the right without a suppression of the maximum response (Arunlakshana & Schild, 1959).

However, the present experiments reveal that the effect of glucagon in attenuating the vasoconstrictor responses of the hepatic arterial vascular bed is not restricted to α-adrenoceptor stimulation by catecholamines, since the antagonism occurred towards the increases in hepatic arterial vascular resistance caused by the arterial injections of the vasoactive polypeptides angiotensin and vasopressin. The attenuation of the vasoconstrictor responses to the test doses of noradrenaline, angiotensin and vasopressin was similar in extent and time-course, whether the glucagon was injected close-arterially in a single dose or infused intra-arterially (Figures 2–4). The attenuation of the responses was highly significant and was present even at the lowest glucagon infusion used (2.5 µg/min) which would, on calculation, have resulted in a maximum hepatic arterial blood concentration of 9.1 ng/ml.

The nature of the antagonism between glucagon and arterial angiotensin was similar to that for noradrenaline/glucagon: a competitive relationship. The interaction between glucagon and specific angiotensin receptors may conform to this pattern, but in addition there is the observation that angiotensin interacts with adrenergic nerve terminals, enhancing and possibly even stimulating the release of the transmitter (Distler, Liebau & Wolff, 1965; Regoli, Park & Rioux, 1974). The interaction of angiotensin and glucagon might, therefore be expected to conform closely to the characteristics of noradrenaline/glucagon interaction if a major component of the action of angiotensin involves an effect on the adrenergic nerve terminal.

The nature of the interaction between vasopressin and glucagon is different: the duration of antagonism following infusions of glucagon was longer than for either noradrenaline or angiotensin, recovery of the vasoconstrictor effects of vasopressin often being slow and somewhat incomplete (Figure 5c). The characteristics of the antagonism, obtained by determination of the complete dose-response curves before, during, and after an infusion of glucagon revealed in each of three experiments a 'noncompetitive' antagonism since there was a nonparallel shift of the dose-response curve to the right with a very marked suppression of the maximum response (Arunlakshana & Schild, 1959).

It is difficult at present to propose mechanisms at the cellular level which might explain these effects and interactions since it is generally necessary to extrapolate observations made in non-vascular cells to the vascular smooth muscle. Glucagon hyperpolarizes the hepatic parenchymal cells with a consequent raising of the excitation threshold (Peterson, 1974). If a similar effect occurred in the vascular smooth muscle, it might cause relaxation of arteriolar tone with vasodilatation. This hyperpolarization could also explain the antagonism to vascular smooth muscle stimulants, all three of which, noradrenaline (Keatinge, 1964), angiotensin (Keatinge, 1966) and vasopressin (Steedman, 1966), depolarize various excitable tissues and might therefore be antagonized by a hyperpolarizing substance.

A simple link between hormones with effects on blood sugar levels and their effects on the hepatic arterial vasculature is improbable on the basis of the results obtained with insulin. A dose of insulin injected acutely to the liver, which was of the same order as the daily requirement of a pancreatectomized man (i.e. about 0.5 iu kg^{-1}: Goldner & Clark, 1944) produced only a modest and very transient hepatic arterial vasoconstriction, and an attenuation of the effects of noradrenaline which was very much smaller in extent and of very much shorter duration of action than that of glucagon.

In states where high circulating levels of vasoconstrictor hormones might be attained, it would be undesirable for there to be hepatic arterial vasoconstriction which would limit the blood flow to the liver at a time when mobilization of hepatic glycogen would be vital. On these grounds, it is reasonable to postulate that the demonstrated antagonism of the vasoconstrictor actions of noradrenaline, angiotensin and vasopressin by glucagon could represent a physiological mechanism for the 'protection' of the hepatic arterial vasculature from the effects of circulating vasoconstrictor substances.

In the present experiments, glucagon and the vasoconstrictor agents were administered close-arterially, whereas under physiological conditions, glucagon enters the liver predominantly by the portal circulation. The interaction between the portal and systemic circulations, and substances which they may contain has not been studied, and is a point requiring further elucidation.

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TRYPTOPHAN METABOLISM IN THE ISOLATED PERFUSED LIVER OF THE RAT: EFFECTS OF TRYPTOPHAN CONCENTRATION, HYDROCORTISONE AND ALLOPURINOL ON TRYPTOPHAN PYRROLASE ACTIVITY AND KYNURENINE FORMATION

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1 The effect of tryptophan concentration on the rate of kynurenine appearance and tryptophan disappearance in the medium perfused through the isolated liver of the rat has been investigated. The effect of pretreatment of the rat with hydrocortisone or allopurinol was also examined, together with the effects of these treatments on liver tryptophan pyrrolase activity measured *in vitro* at the beginning and end of perfusion.

2 Hydrocortisone (5 mg/kg) injection 3 h before perfusion resulted in a four-fold increase in kynurenine production by the liver during perfusion with a medium containing either 0.1 mmol/l or 1.0 mmol/l tryptophan. Injection of allopurinol (20 mg/kg) together with hydrocortisone and addition of allopurinol (4 mg/100 ml) to the medium abolished the hydrocortisone-induced rise of kynurenine in the 0.1 mmol/l tryptophan medium but not the 1.0 mmol/l tryptophan medium.

3 Injection of cycloheximide (30 mg/kg) with hydrocortisone (5 mg/kg) 3 h before perfusion inhibited the hydrocortisone-induced rise of kynurenine production and the increase in pyrrolase activity measured *in vitro* both before and at the end of perfusion with 1.0 mmol/l tryptophan. This last result suggests that protein synthesis is involved not only in hydrocortisone induction of pyrrolase but also in substrate induction.

4 Kynurenine production in the 1.0 mmol/l tryptophan medium was less in both saline- and hydrocortisone-treated older rats (335–450 g) compared to younger rats (180–220 g). In agreement with a previous study, pyrrolase activity *in vitro* was also lower in both saline- and hydrocortisone-treated older rats at the beginning of the perfusion although activity had risen equally in both young and older rats at the end of perfusion.

5 There was little correlation between the rate of tryptophan disappearance from the medium and the activity of tryptophan pyrrolase either as measured *in vitro* or as indicated by the rate of kynurenine production.

6 In general, the production of kynurenine in the medium at the end of the 60 min perfusion was indicative of *in vitro* pyrrolase activity at the start of the perfusion.

7 It is concluded that while *in vitro* pyrrolase assay does not give a quantitative index of kynurenine production, it does provide a qualitative index. Furthermore, if kynurenine production in the isolated perfused liver of the rat is indicative of *in vivo* pyrrolase activity, then hydrocortisone must induce pyrrolase activity *in vivo*.

Introduction

The liver enzyme tryptophan pyrrolase (L-tryptophan-2, 3-dioxygenase, EC1.13.1.12) is the first enzyme on the major metabolic pathway degrading tryptophan (Figure 1). Its activity is increased by corticosteroids and tryptophan (Knox, 1951) and inhibited by allopurinol (Becking & Johnson, 1967; Green & Curzon, 1968; Badawy & Evans, 1973). Recent evidence has suggested that when the activity of the enzyme is increased by hydrocortisone treatment, plasma, liver and brain tryptophan concentrations

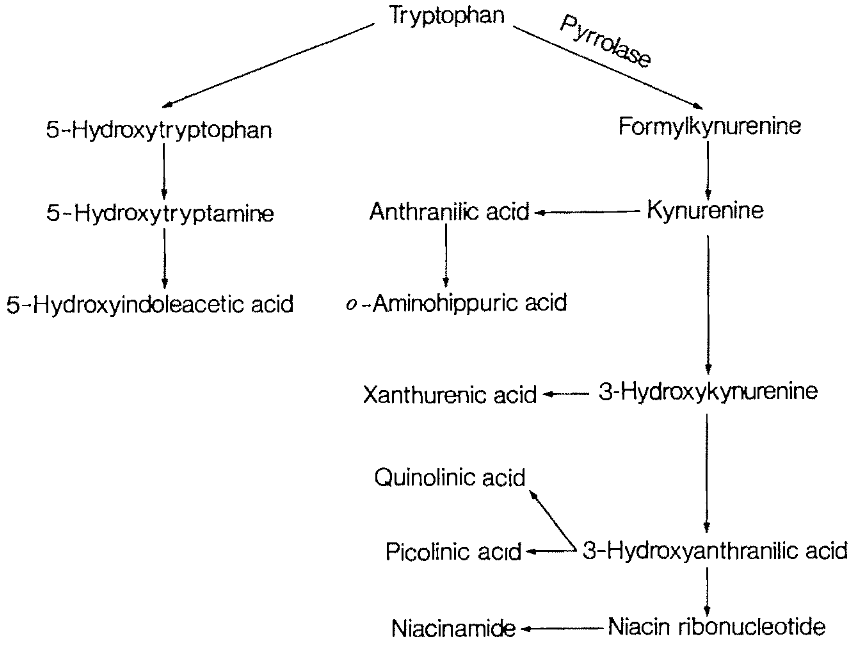

Figure 1 Tryptophan metabolism. A simplified diagrammatic metabolic pathway.

decrease and the brain tryptophan change leads to a decreased rate of synthesis of the neurotransmitter 5-hydroxytryptamine (Green, Sourkes & Young, 1975b; Green, Woods, Knott & Curzon, 1975c), the product of another metabolic pathway of tryptophan (Figure 1).

One difficulty in interpreting previous data on the effect of drugs on pyrrolase activity is that the compounds were administered *in vivo* and subsequent measurement of pyrrolase activity was performed *in vitro*. To avoid this problem, Kim & Miller (1969) measured pyrrolase activity in the isolated perfused liver of the rat. However, when they perfused for 6 h, adding hydrocortisone to the medium, they found few significant changes of pyrrolase activity in terms of either tryptophan removal from the medium or appearance of kynurenine, the product of pyrrolase activity. This is at variance with *in vivo* studies which have demonstrated that steroids and tryptophan produce significant alterations in the rate of expired $^{14}CO_2$ from DL-[ring 2-^{14}C]-tryptophan (Moran & Sourkes, 1963; Green, 1969; Green *et al.*, 1975b; Young & Sourkes, 1975). Kim & Miller (1969) however were also unable to find a statistically significant increase in $^{14}CO_2$ excretion using these techniques.

We have now investigated the problem of the *in vivo* activity of this enzyme by observing the rate of disappearance of tryptophan and appearance of kynurenine in the medium circulating through an isolated perfused liver of the rat. The results are compared with the activity of the enzyme measured *in vitro* in a lobe obtained from the perfused liver before the start and at the end of the perfusion.

A preliminary account of some of this work has been given to the British Pharmacological Society (Green, Joseph & Woods, 1975a).

Methods

All experiments were performed on adult male Sprague-Dawley rats weighing 180–220 g (Anglia Laboratory Animals Ltd, Alconbury, Huntingdon) except those involved in the study on the effect of age, when rats weighing 335–450 g were used.

Rats were anaesthetized with pentobarbitone sodium (Abbott, Queensborough, Kent), 60 mg/kg intraperitoneally, and livers perfused by the method of Hems, Ross, Berry & Krebs (1966) using a semi-synthetic medium (Woods, Eggleston & Krebs, 1970) consisting of dialysed bovine serum albumin (2.6 g/100 ml), washed aged human red cells (2.5 g/100 ml) and bicarbonate buffered saline (Krebs & Henseleit, 1932) containing glucose (5 mmol/litre). During perfusion the medium was gassed with 95% O_2 and 5% CO_2 at a rate of 150–200 ml/minute. Before the start of perfusion, a small piece of liver was tied off and removed for measurement of pyrrolase activity *in vitro*. A second piece was removed at the end of perfusion for further measurement of *in vitro* pyrrolase activity.

Pyrrolase activity was measured in the liver lobes by the method of Knox & Auerbach (1955) after the addition of 2 μM haematin to the reaction mixture (Kevitz & Wagner, 1965). *In vitro* pyrrolase activity is expressed in terms of units of activity, one unit being 1 μmol kynurenine formed h^{-1} g^{-1} liver (dry weight) at 37°C.

Livers were perfused at a rate of between 2 and 3 ml/min per g tissue with 100 ml of medium. A 5 ml sample was removed at zero time for determination of tryptophan and kynurenine and 5 ml samples removed at 15, 30, 45 and 60 min for further determinations. Results have been calculated allowing for the removal of these samples. The concentration of tryptophan is expressed in terms of μg compound per ml perfusate plasma. Plasma refers to the medium after centrifugation to remove red blood cells.

Tryptophan was measured both in medium and liver by the method of Denckla & Dewey (1967). A new method was developed to measure kynurenine in the medium as it was found that tryptophan seriously interfered with the method of Spiera & Vallarino (1969). The method developed is based on the principle that after deproteinization, kynurenine can be diazotized and coupled with napthyl ethylene diamine, the coloured product then being extracted into amyl alcohol and back-extracted into dilute sulphuric acid to be read at 560 nM on a spectrophotometer (Joseph, 1973).

The perfusion medium (5 ml) was centrifuged and 3 ml of the 'plasma' supernatant deproteinized with 3 ml trichloroacetic acid (20% v/v). After centrifugation, 5 ml of the supernatant was pipetted into a 25 ml stoppered tube, and 0.1 ml sodium nitrite (0.5 M) added and mixed. After 5 min, 0.1 ml ammonium sulphamate (2.5%) was added and mixed and after a further 5 min, 0.2 ml napthyl ethylene diamine (0.25%) in ethanol (95% v/v) was also added and mixed. One hour later amyl alcohol (5 ml) was added, and the tubes stoppered, shaken for 1 min and centrifuged briefly; 4 ml of the upper organic phase was transferred to a 25 ml stoppered tube containing 4 ml sulphuric acid (1N). The tube was shaken for 1 min, centrifuged briefly and 3 ml of the lower aqueous phase pipetted into a test tube and clarified with acetone (0.2 ml). Optical density was read on a Unicam SP1800 spectrophotometer. Standards of 5 ml containing 1 μg/ml kynurenine sulphate in 10% (v/v) trichloroacetic acid and blanks of 5 ml trichloroacetic acid (10% v/v) were carried through the procedure in place of deproteinized 'plasma' supernatant.

No interference resulted when tryptophan, or other amino acids, 3-hydroxykynurenine, kynurenic acid, 3-hydroxyanthranilic acid or *p*-aminobenzoic acid were added to 'plasma'. Acetylkynurenine and *o*-aminohippuric acid did interfere, giving 80% and 50% respectively of the reading obtained with an equimolar solution of kynurenine (Joseph & Risby, 1975; Friedel & Curzon, unpublished observations).

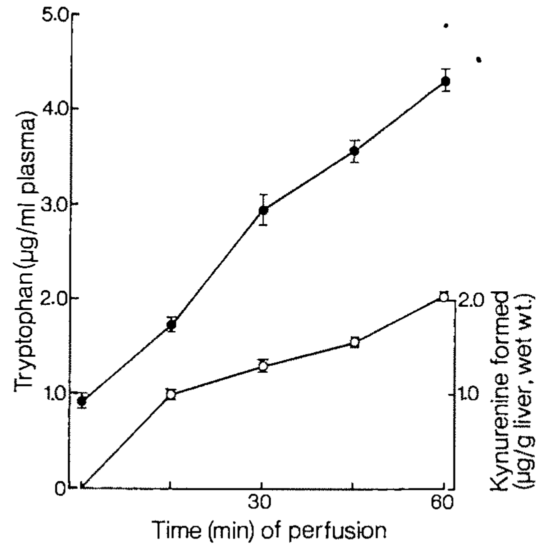

Figure 2 Effect of perfusion of basal medium on the production of kynurenine and concentration of tryptophan in the medium. Control rat livers were perfused with the basal medium and tryptophan (●) and kynurenine (○) production measured in the medium. Points show mean of determinations on 4 animals. Vertical bars show s.e. mean.

Plasma 5-hydroxytryptamine and 5-hydroxyindoleacetic acid were measured essentially by the method of Curzon & Green (1970).

Drugs were administered 3 h before the perfusion. They were given intraperitoneally, dissolved in 0.9% w/v NaCl solution (saline) or, in the case of allopurinol, suspended in saline containing 1% carboxymethyl cellulose. They were obtained from the following sources: L-tryptophan from Sigma Chemical Co., hydrocortisone sodium succinate (Solu-cortef) from Upjohn Ltd and allopurinol from Burroughs Wellcome.

Results

Effects of perfusion of basal medium on kynurenine and tryptophan concentration in the medium and on pyrrolase activity

Rats were anaesthetized and the livers perfused with basal medium with no tryptophan added to it. There was a small but significant appearance of kynurenine

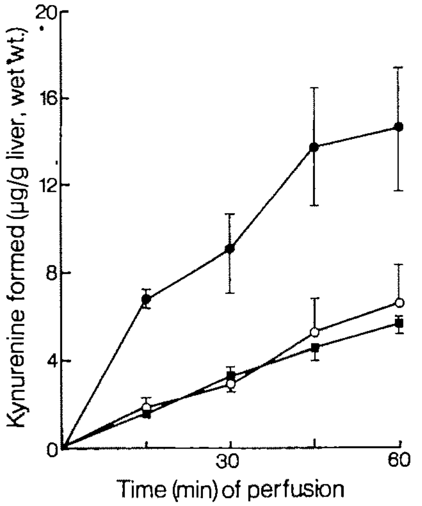

Figure 3 Effect of perfusion of 0.1 mmol/l tryptophan medium on the production of kynurenine in perfusate from livers of rats injected 3 h previously with saline (O), hydrocortisone 5 mg/kg (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) also added to the perfusate (■). Points show mean of determinations performed on at least 3 animals. Vertical bars show s.e. mean. When allopurinol was injected with hydrocortisone as above but not added to the medium the final kynurenine formation was 10.13 ± 1.33 (3).

over the 60 min perfusion period (Figure 2). The basal medium contained 0.95 μg/ml tryptophan and, during perfusion, its tryptophan concentration rose linearly to reach a concentration of 4.34 μg/ml (Figure 2). *In vitro* pyrrolase activity was essentially unchanged at the end of the 60 min perfusion compared with activity at the start of perfusion (Table 1).

Effects of hydrocortisone and allopurinol on the production of kynurenine by the isolated perfused liver

Rats were injected with saline, hydrocortisone (5 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg). Three hours later they were anaesthetized and the livers perfused with the medium containing either 0.1 mmol/l (20.4 μg/ml) or 1.0 mmol/l (204 μg/ml) tryptophan.

Table 1 Effect of perfusion of basal medium on *in vitro* pyrrolase activity

Treatment	*Pyrrolase activity (units)*
Control	3.62 ± 1.78 (4)
60 min perfusion with basal medium	3.96 ± 0.55 (4)

Results expressed as mean ± s.e. mean with number of animals shown in brackets. Pyrrolase activity expressed as units; for details, see methods section.

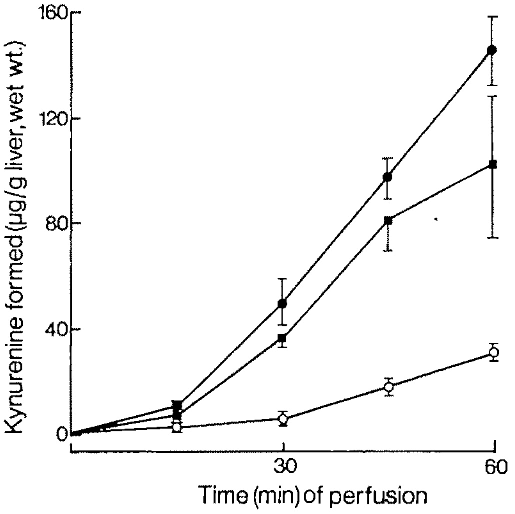

Figure 4 Effect of hydrocortisone and hydrocortisone plus allopurinol on kynurenine production in the perfusion medium. Effect of perfusion of 1.0 mmol/l tryptophan medium on the production of kynurenine in perfusate from livers of rats injected 3 h previously with saline (O), hydrocortisone 5 mg/kg (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) also added to the perfusate (■). Points show mean of determinations performed on at least 3 animals. Vertical bars show s.e. mean. When allopurinol was injected with hydrocortisone as above but not added to the medium the final kynurenine formation was 99.3 ± 15.06 (3).

When 0.1 mmol/l tryptophan was perfused kynurenine appearance in the medium increased during the second half of the perfusion (Figure 3). A similar pattern was observed when 1.0 mmol/l tryptophan was perfused (Figure 4). When hydrocortisone (5 mg/kg) had been injected 3 h prior to perfusion, considerably more kynurenine appeared in the medium during perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan.

When allopurinol (20 mg/kg) was injected with the hydrocortisone (5 mg/kg), it brought about only a slight reduction in the increased kynurenine production which had been caused by the hydrocortisone. This was observed following perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan (see legends to Figures 3 and 4). However, if rats that had been pretreated with hydrocortisone plus allopurinol were perfused with a medium containing 0.1 mM tryptophan plus allopurinol (4 mg/100 ml medium), the concentration of kynurenine in the medium was no greater than in the control animals (Figure 3). When 1.0 mmol/l tryptophan was perfused through livers of rats pretreated with hydrocortisone plus allopurinol, there was little inhibition of the hydrocortisone-induced rise of kynurenine production even if allopurinol (4 mg/100 ml medium) had been added to the medium (Figure 4).

When livers from rats pretreated with allopurinol (20 mg/kg) were perfused with 0.1 mmol/l tryptophan plus allopurinol (4 mg/100 ml medium) kynurenine production was no lower than that seen in animals not given allopurinol.

Effect of hydrocortisone and allopurinol on rat liver pyrrolase activity measured in vitro *before and after the 60 min perfusion*

As stated in the methods section, a lobe of liver was tied off both at the beginning and the end of the perfusion and pyrrolase activity measured *in vitro* on these lobes. This section gives the results of these measurements performed on rats treated as described in the last section.

When control (untreated) rat livers were perfused with 0.1 mmol/l tryptophan there was a small increase in pyrrolase activity (50%) at the end of the 60 min perfusion (Table 2). When 1.0 mmol/l tryptophan was present, activity had increased 300% at the end of perfusion (Table 2).

Injection of hydrocortisone (5 mg/kg) 3 h before the perfusion, as expected, increased initial pyrrolase activity. There was no further increase in enzyme activity at the end of the 60 min perfusion with 0.1 mmol/l tryptophan, but a considerable further increase when 1.0 mmol/l tryptophan had been perfused.

If rats had been injected with hydrocortisone (5 mg/kg) and allopurinol (20 mg/kg) 3 h previously, there was a significant inhibition of the rise of activity seen after hydrocortisone alone (Table 2). While this was overcome at the end of the perfusion with 0.1 mmol/l tryptophan alone, addition of allopurinol to the medium prevented this increase. Allopurinol (4 mg/100 ml) in the medium did not, however, prevent the increase seen when 1.0 mmol/l tryptophan was perfused (Table 2).

Injection of animals with allopurinol (20 mg/kg) produced a 30% inhibition of enzyme activity 3 h later and there was a further small inhibition of activity at the end of perfusion when allopurinol (4 mg/100 ml medium) was added to the 0.1 mmol/l tryptophan medium (Table 2).

Table 2 Pyrrolase activity before and at the end of a 60 min perfusion with 0.1 mmol/l or 1.0 mmol/l tryptophan medium through livers of rats pretreated with either hydrocortisone, allopurinol or hydrocortisone plus allopurinol

Injected	*Pyrrolase activity (units) before perfusion*	*Added to medium*	*Pyrrolase activity (units) at end of 60 min perfusion with:*	
			0.1 mmol/l tryptophan	*1.0 mmol/l tryptophan*
Saline	6.32 ± 0.75 (13)	—	9.54 ± 3.16 (5)	20.7 ± 6.75 (3)
Hydrocortisone (5 mg/kg)	20.15 ± 2.20 (21)*	—	22.9 ± 3.54 (6)*	48.7 ± 3.52 (4)*
Hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg)	10.54 ± 1.85 (10)†	—	19.0 ± 6.76 (3)	35.2 ± 6.7 (3)
		Allopurinol	9.91 ± 3.64 (3)†	43.0 ± 6.2 (3)
Allopurinol (20 mg/kg)	4.04 ± 0.40 (3)‡	Allopurinol	3.41 ± 1.03 (3)‡	ND

Rats were injected with saline, hydrocortisone (5 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg), 3 h before perfusion of the livers with 0.1 mmol/l or 1.0 mmol/l tryptophan. At the start and end of the perfusion, pyrrolase activity was determined and activity is given in units (see methods section). Allopurinol (4 mg/100 ml) was added to some of the perfusion media as shown above. Results expressed as mean ± s.e. mean with number of observations in brackets. * Different from saline-injected controls, $P<0.001$. † Different from hydrocortisone-injected animals, $P<0.001$. ‡ Different from saline-injected controls, $P<0.05$. ND not determined.

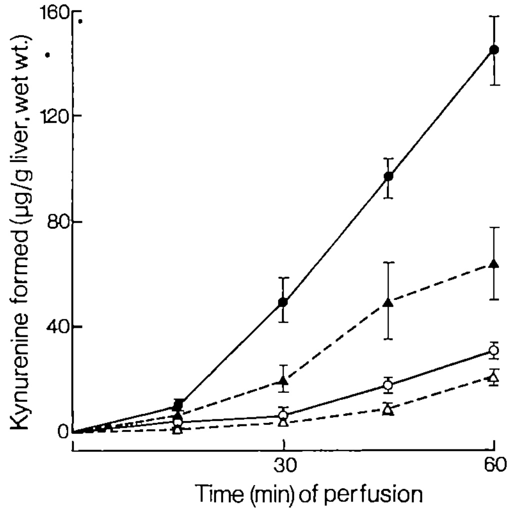

Figure 5 Effect of hydrocortisone and age of the rats on production of kynurenine in the liver perfusion medium. Rats were injected with either saline or hydrocortisone (5 mg/kg) 3 h prior to perfusion with 1.0 mmol/l tryptophan medium. Young animals approx. 35 days (solid lines) and older rats approx. 100 days (broken lines) were used. Open symbols show kynurenine production in perfusate of control animals. Solid symbols show concentration of kynurenine in perfusate of animals injected with hydrocortisone (5 mg/kg) 3 h previously. Points show mean of determinations performed on at least 3 animals with ±s.e.m. shown as vertical bars. At 60 min, old rats have a lower production of kynurenine than younger rats injected with saline ($P < 0.05$) or hydrocortisone ($P < 0.01$).

Effect of age of the rats on appearance of kynurenine in medium during perfusion with 1.0 mmol/l tryptophan medium and on in vitro *pyrrolase activity*

The previous observation that older rats have a lower *in vitro* basal pyrrolase activity and show a smaller increase in activity following hydrocortisone (Green & Curzon, 1975) was confirmed (Table 3). At the end of the 60 min perfusion with 1.0 mmol/l tryptophan, there was a rise in *in vitro* pyrrolase activity both in control and hydrocortisone-treated rats. which was similar in young and older animals (Table 3).

During perfusion with 1.0 mmol/l tryptophan medium, there was a small, but statistically significant, decrease in the amount of kynurenine appearing in the medium from older animals compared with younger animals (Figure 5) and a much larger decrease when both groups had been pretreated with 5 mg/kg hydrocortisone (Figure 4).

Effect of cycloheximide on hydrocortisone-induced increase of pyrrolase activity

We wished to observe what would happen if a protein synthesis inhibitor was given with the hydrocortisone to confirm that hydrocortisone induction of pyrrolase activity is the result of new enzyme protein being formed. Rats were injected with saline, hydrocortisone (5 mg/kg) or hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg) 3 h before perfusion with the 1 mmol/l tryptophan medium. *In vitro* pyrrolase activity was measured, as before, at the beginning and end of perfusion.

Cycloheximide totally abolished the hydrocortisone-induced rise in pyrrolase activity observed at the beginning of perfusion. Furthermore, there was little increase in *in vitro* pyrrolase activity at the end of 1 h perfusion (Table 4). The cycloheximide pretreatment also abolished the increase of kynurenine in the medium observed when hydrocortisone alone had been injected (Table 4).

Concentration of tryptophan in the medium during liver perfusion of rats pretreated with hydrocortisone, allopurinol, hydrocortisone plus allopurinol and hydrocortisone plus cycloheximide

The concentration of tryptophan in the perfusion medium used during the experiments already

Table 3 Effect of hydrocortisone and age of rats on pyrrolase activity before and after a 60 min perfusion with 1.0 mmol/l tryptophan medium

Age (days)	*Injected*	*Pyrrolase activity (units):*	
		before perfusion	*after perfusion*
35	Saline	6.32 ± 0.75 (13)	20.7 ± 6.75 (3)
100	Saline	4.12 ± 1.60 (3)	21.4 ± 0.44 (3)
35	Hydrocortisone	20.15 ± 2.20 (21)	48.7 ± 3.52 (4)
100	Hydrocortisone	10.03 ± 2.96 (3)*	42.8 ± 5.7 (3)

Rats were injected with saline or hydrocortisone (5 mg/kg) 3 h before perfusion. Pyrrolase activity determined at start of perfusion and at end of 60 min perfusion with 1.0 mmol/l tryptophan medium. Pyrrolase activity expressed in units (see methods section). Results expressed as mean ± s.e. mean with number of observations in brackets. * Different from 35 day hydrocortisone-treated rats, $P < 0.01$.

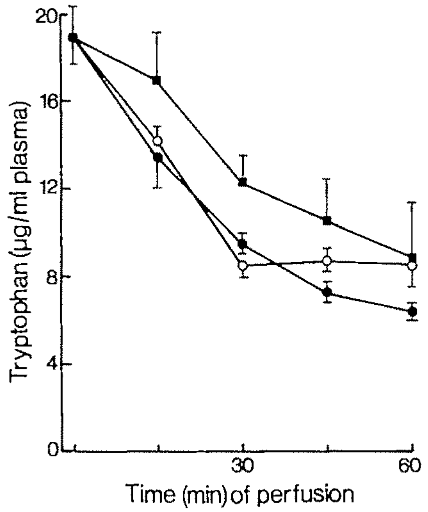

Figure 6 Concentration of tryptophan in medium during a 60 min incubation; effect of hydrocortisone or hydrocortisone plus allopurinol pretreatment. The change in tryptophan concentration during perfusion with 0.1 mmol/l tryptophan medium is shown. Rats were injected 3 h before the start of perfusion with saline (O), hydrocortisone (5 mg/kg) (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) added to the perfusion medium (■).

described was also studied. The decline in tryptophan concentration in the 0.1 mmol/l tryptophan medium was very similar in the perfusate from livers of rats pretreated either with hydrocortisone (5 mg/kg) or saline. There was a slower initial decline in tryptophan concentration in the perfusate from livers of rats pretreated with hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) provided that allopurinol (4 mg/100 ml) had also been added to the medium (Figure 6). However, the tryptophan concentration at 1 h was no different from that found in the control animals.

When 1.0 mmol/l tryptophan was perfused through livers of rats pretreated with hydrocortisone (5 mg/kg), there was an initial rapid drop in tryptophan concentration which was also seen in saline-injected controls. However, while the tryptophan concentration in the perfusate of hydrocortisone-treated rats continued to decline steadily, that of the control animals decreased only slightly during the remaining 45 min (Figure 7). The decrease in tryptophan concentration in the perfusate from livers of rats pretreated with hydrocortisone (5 mg/kg) and allopurinol (20 mg/kg), even with allopurinol (4 mg/100 ml) in the medium, showed little difference from the perfusate of rats injected with hydrocortisone only (Figure 7).

Injection of cycloheximide (30 mg/kg) with hydrocortisone (5 mg/kg) was effective in preventing the additional tryptophan decrease in the 1 mmol/l tryptophan perfusate produced by hydrocortisone rather than saline (Table 5).

The concentration of tryptophan at the end of perfusion through livers of 100 day old rats was lower than when 35 day rats were used (Table 5). The further decrease in tryptophan concentration produced by pretreatment with hydrocortisone (5 mg/kg) compared to saline-injected rats was also greater in older rats (Table 5).

Concentration of tryptophan in the liver 30 min after the start of perfusion with a medium containing 0.1 mmol/l or 1.0 mmol/l tryptophan

A lobe of liver was tied off at the start of perfusion and the tryptophan concentration measured. Thirty

Table 4 Effect of cycloheximide on the hydrocortisone-induced rise of pyrrolase activity and production of kynurenine in the perfusion medium of livers perfused with 1.0 mmol/l tryptophan

Injected	*Kynurenine in medium at end of 60 min perfusion (µg ml^{-1} g^{-1} liver, wet wt)*	*Pyrrolase activity (units):* before perfusion	at end of perfusion
Saline	31.1 ± 3.1 (3)	6.32 ± 0.75 (13)	20.7 ± 6.75 (3)
Hydrocortisone (5 mg/kg)	145.0 ± 13.3 (8)*	20.15 ± 2.20 (21)†	48.7 ± 3.52 (4)†
Hydrocortisone (5 mg/kg) plus Cycloheximide (30 mg/kg)	32.4 ± 8.7 (4)‡	6.53 ± 0.67 (4)§	10.2 ± 2.8 (4)§

Results show production of kynurenine at end of 60 min perfusion and pyrrolase activity measured *in vitro* at beginning and end of perfusion with 1.0 mmol/l tryptophan. Rats were injected with saline, hydrocortisone or hydrocortisone plus cycloheximide 3 h before perfusion. Results are expressed as mean ± s.e. mean with number of determinations in brackets. *Different from saline, $P<0.05$. †Different from saline, $P<0.01$. ‡Different from hydrocortisone, $P<0.05$. §Different from hydrocortisone, $P<0.01$.

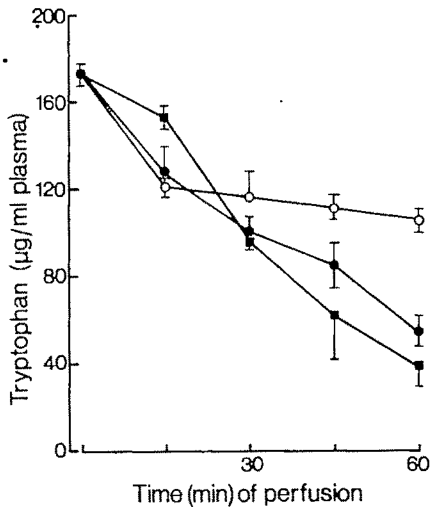

Figure 7 Concentration of tryptophan in medium during a 60 min incubation; effect of hydrocortisone or hydrocortisone plus allopurinol pretreatment. The change in tryptophan concentration during perfusion with 1.0 mmol/l tryptophan medium is shown. Rats were injected 3 h before the start of perfusion with saline (○), hydrocortisone (5 mg/kg) (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) added to the perfusion medium (■).

minutes later following perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan a further lobe of liver was taken and tryptophan concentration in these lobes also measured. While tryptophan was not concentrated by the liver during perfusion with 0.1 mmol/l tryptophan, there was some tryptophan uptake during perfusion with 1.0 mmol/l tryptophan (Table 6).

Discussion

The first major point to be clarified is whether the appearance of kynurenine in the perfusion medium is indicative of tryptophan pyrrolase activity. Tryptophan pyrrolase is the first enzyme on the kynurenine pathway (Figure 1) converting L-tryptophan to L-formylkynurenine (Knox & Mehler, 1950) which is metabolized rapidly to kynurenine by formylase, an enzyme present in excess in the liver (Mehler & Knox, 1950). The normal metabolite of pyrrolase activity detected in the *in vitro* assay therefore is kynurenine.

Kynurenine is metabolized to other metabolites detectable in urine after a tryptophan load to various species, including rat and man (Brown & Price, 1956; McDaniel, Hundley & Sebrell, 1956; Korbitz, Price & Brown, 1963; Leklem, Woodford & Brown, 1969; Leklem, 1971).

In our experiments, the mean tryptophan loss from

Table 5 Concentration of tryptophan in medium at end of 60 min perfusion (with 1.0 mmol/l or 0.1 mmol/l tryptophan) of livers from rats of different ages pretreated with hydrocortisone, hydrocortisone plus allopurinol, allopurinol or hydrocortisone plus cycloheximide

Age (days)	*Injected (3 h previously)*	*Added to medium*	*Tryptophan conc (μg/ml plasma) 60 min after start of perfusion with:*	
			0.1 mmol/l tryptophan	*1.0 mmol/l tryptophan*
35	Saline	—	8.5 ±0.6 (3)	106.0± 5.6 (3)
35	Hydrocortisone (5 mg/kg)	—	6.32±0.34 (5)	54.0± 7.0 (6)*
35	Hydrocortisone (5 mg/kg)	—	6.71±1.5 (3)	50.0± 6.0 (3)
	plus allopurinol (20 mg/kg)	Allopurinol	8.8 ±2.5 (3)	39.0±10.0 (3)
35	Allopurinol (20 mg/kg)	Allopurinol	10.0 ±1.8 (3)	ND
35	Hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg)	—	ND	116.0±15.0 (3)†
100	Saline	—	ND	34.0± 6.0 (3)
100	Hydrocortisone (5 mg/kg)	—	ND	8.6± 3.0 (3)

Rats were injected 3 h before the start of perfusion with either saline, hydrocortisone (5 mg/kg), hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg). In some experiments allopurinol (4 mg/100 ml) was added to the medium as shown above. In another group of experiments, 100 day old rats were pretreated with saline or hydrocortisone (5 mg/kg) for comparison with younger rats. Results show the concentration of tryptophan in the medium at the end of a 60 min perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan medium. Results expressed in μg tryptophan/ml plasma and show mean s.e. mean with number of determinations in brackets. ND = not determined. *Different from saline-injected, $P<0.01$. †Different from hydrocortisone-injected, $P<0.01$.

the medium containing 1 mmol/l tryptophan in the first 30 min of perfusion was 6700 µg; 292 µg had accumulated in liver (Table 6) and a further 63 µg was recovered as kynurenine in the medium. Thus out of 20.4 mg of tryptophan added to the medium 5.345 mg is unaccounted for (25%). Kynurenine is only a small proportion of the total metabolites of the kynurenine pathway found in urine after a tryptophan load (see review by Leklem, 1971) so it seems reasonable to assume that the tryptophan not accounted for is present as other metabolic products. No evidence was found for the presence of 5-hydroxytryptamine or 5-hydroxyindoleacetic acid in the medium under our conditions. When similar calculations were applied to our studies using 0.1 mmol/l tryptophan it was found that there was a recovery of somewhat more than 50% of the tryptophan after 30 minutes.

Clearly kynurenine can only reflect the state of flux down the pathway and is not the sole product of pyrrolase activity. Nevertheless, it seems to be an index of tryptophan degradation, a view strengthened by the fact that little kynurenine appears in the medium in the absence of tryptophan in the perfusate (basal medium).

The method for kynurenine determination is not completely specific (see methods section). However, the known interfering compounds are other products on the pyrrolase pathway, so that the compounds being measured as kynurenine almost certainly represent an index of pyrrolase activity. During the final stages of this investigation, a more specific method for determining plasma kynurenine was developed (Joseph & Risby, 1975). It produced qualitatively similar results when applied to some of the samples in this study, although the actual kynurenine values were about 50% lower.

The observation that the tryptophan in the medium rose during perfusion with the basal medium suggests that the liver releases tryptophan either from endogenous stores or protein degradation when the circulating amino acid concentration is low. This also occurs with some other amino acids (Schimassek & Gerok, 1965). It is not known however whether a similar release occurs when tryptophan is present in the medium. Exchange diffusion of tryptophan has certainly been observed in brain tissue (Parfitt & Grahame-Smith, 1974).

Kynurenine concentration increased to some extent during the second half of the perfusion with 0.1 mmol/l tryptophan and this observation, taken together with the increase in enzyme activity at the end of the perfusion measured *in vitro* (Table 2) and the rapid drop in plasma tryptophan during the first 30 min suggests some degree of enzyme induction. This is not too surprising if one assumes that plasma free tryptophan only is directly available to the liver (for a discussion of the role of free and bound tryptophan, see Curzon & Knott, 1974). A perfusate tryptophan concentration of 0.1 mmol/l is somewhat higher than that found under physiological conditions and since albumin is only at half physiological concentration more free tryptophan is available than normal to induce the enzyme. It may be that plasma tryptophan concentration becomes steady at 8 µg/ml which would now correspond to a physiological free tryptophan concentration. This further suggests that tryptophan is only metabolized by pyrrolase when the concentration of unbound amino acid is greater than normal, thereby assisting tryptophan economy when necessary.

Although Kim & Miller (1969) were unable to observe any increase in kynurenine concentration in the perfusion media of rats treated with hydrocortisone compared with control animals, we observed a four-fold increase both during perfusion with 0.1 mmol/l and 1.0 mmol/l tryptophan. This difference is probably attributable to the different technique employed. We began to perfuse when the enzyme had already been induced but Kim & Miller perfused the liver for several hours and then added hydrocortisone to the medium. Since steroid induction of pyrrolase involves the formation of new enzyme protein (Feigelson & Greengard, 1962) it is not, perhaps, surprising that little new enzyme protein was formed in livers perfused with a rabbit blood medium (John & Miller, 1966) which may have been deficient

Table 6 Concentration of tryptophan in the isolated perfused rat liver 30 min after start of perfusion

Time after start of perfusion (min)	*Concentration of tryptophan in medium (mmol/l)*	*Concentration of tryptophan in liver (µg/g liver, wet wt)*	*Total tryptophan accumulated by liver (µg)**
0	—	8.65 ± 0.92 (6)	—
30	0.1	9.80 ± 1.20 (3)	13.8 ± 3.2 (3)
30	1.0	48.93 ± 8.68 (4)	292 ± 53.4 (4)

* Calculated on basis of initial and final concentration of tryptophan in liver of individual experiments multiplied by the liver weight of that experiment. Results show mean ± s.e. mean with number of observations in brackets. Concentration of tryptophan in liver at 0 min was performed before the start of experiment. There is some error in these results deriving from plasma tryptophan trapped in the blood vessels of the liver. Extracellular space in the liver has been calculated at 22.2% (Woods, Eggleston & Krebs, 1970).

in some of the factors required for synthesizing new enzyme. The present results demonstrate clearly that hydrocortisone increases enzyme activity in the intact organ. This effect is apparent during subsequent perfusion and is consistent with previous observations demonstrating an increase in pyrrolase activity *in vivo* by measurement of the rate of degradation of labelled tryptophan (Green *et al.*, 1975b; Young & Sourkes, 1975).

Cycloheximide blocked the hydrocortisone-produced increase in kynurenine formation; other protein synthesis inhibitors have been shown to have similar effects (Gray, Camiener & Bhuyan, 1964; Valyi-Nagy & Doroczy, 1967). While nogalamycin does not alter substrate induction (Gray *et al.*, 1964), Korner & Labrie (1967) showed that actinomycin inhibited substrate induction. Cycloheximide also inhibited substrate induction occurring during the perfusion (Table 5) which argues against the view that tryptophan induces pyrrolase solely through substrate stabilization (Shimke, Sweeney & Berlin, 1964). Perfusion with 1.0 mmol/l tryptophan increased *in vitro* enzyme activity at the end of perfusion, both in control and hydrocortisone-pretreated animals. This increase was not affected by age, a finding consistent with the fact that substrate induction is not altered in older rats (Correll, Turner & Haining, 1965; Haining & Correll, 1966).

The mechanism by which allopurinol produces its inhibitory action on tryptophan pyrrolase *in vivo* and *in vitro* has been the subject of some debate (see Chytil, 1968; Becking & Johnson, 1969; Julian & Chytil, 1970). Badawy & Evans (1973) demonstrated that allopurinol prevented conjugation of the apoenzyme with its haem activator. In our study allopurinol inhibited the hydrocortisone-induced rise of kynurenine formation but was only really effective when allopurinol was also added to the medium (see Figures 3 and 4, and results in legends), suggesting that it was otherwise being 'washed out' of the liver and inhibition occurred at physiological concentrations of tryptophan (0.1 mmol/l) but not under load conditions (1.0 mmol/l).

There has been discussion as to whether allopurinol might prove useful in preventing breakdown of tryptophan in the liver when the amino acid is given to depressed patients (Badawy & Evans, 1973, 1974; Young & Sourkes, 1974; Fernando, Joseph & Curzon, 1975). To find out whether allopurinol was not inhibiting pyrrolase at higher tryptophan concentrations because its concentration was too low we injected rats with 40 mg/kg allopurinol with hydrocortisone, also adding 10 mg/100 ml to the 1.0 mmol/l tryptophan medium. Even at this high dose of allopurinol, there was no significant decrease in kynurenine production by the isolated perfused liver. While these results indicate that allopurinol may be ineffective as an inhibitor of pyrrolase when a tryptophan load is given, it should be pointed out that Fernando *et al.* (1975) did observe that allopurinol administration increased the concentration of tryptophan in the brain following a tryptophan load. Further, 1 mmol/l is considerably higher than the plasma tryptophan concentration observed in subjects given a 50 mg/kg load (Green & Woods, unpublished observations).

In agreement with Kim & Miller (1969), the rate of tryptophan disappearance from the medium had little connection with previous drug treatment. However, the rate of tryptophan disappearance from the 1.0 mmol/l tryptophan medium was about 10 times faster than from the 0.1 mmol/l tryptophan medium, which is consistent with the fact that this 10-fold increase in tryptophan concentration also resulted in a 10-fold increase in the appearance of kynurenine. The rate of tryptophan disappearance from the 0.1 mmol/l tryptophan medium up to 30 min and the subsequent levelling off is very similar to that observed by Ng, Hagino, Swan & Henderson (1970). Both Kim & Miller (1969) and Ng *et al.* (1970) reported that under load conditions the liver initially removed around 2 mg tryptophan/g liver in one hour. Our results show an initial rate of removal of 2.4 mg tryptophan/g liver in one hour.

The liver perfusion experiments described, clearly do not represent an *in vivo* situation; for example, the medium lacks the presence of other amino acids that compete with tryptophan for uptake into the liver. Nevertheless as a perfused whole organ it is nearer to the *in vivo* situation than a liver homogenate preparation.

In general, there was good correlation between kynurenine appearance in the medium and enzyme activity measured *in vitro* at the beginning of the perfusion. For example, hydrocortisone caused a rise in enzyme activity and kynurenine production by the same factor (four-fold) and allopurinol and cycloheximide reduced the increase in enzyme activity and blocked the increase in kynurenine formation.

The *in vitro* results following hydrocortisone show a kynurenine production of around 20 μmol h^{-1} g^{-1} (dry weight). Conversion of this figure to μg kynurenine h^{-1} g^{-1} (wet weight) using the wet/dry wt ratio of 3.45 (Woods & Krebs, 1971) reveals a value of 1205 μg h^{-1} g^{-1}. Kynurenine production by the isolated perfused liver was 20 μg h^{-1} g^{-1}. Even allowing for the fact that *in vitro* the tryptophan concentration is 3.0 mmol/l whereas it is 1.0 mmol/l in the perfusion, and the fact that the kynurenine is perhaps metabolized further in the perfusion system, these figures reveal that kynurenine production in the 'ideal' situation of the *in vitro* assay with added co-factors bear little relationship in quantitative terms to the *in vivo* situation although as discussed earlier it appears to give a qualitative index of *in vivo* tryptophan pyrrolase activity.

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MECHANISM OF THE INDIRECT SYMPATHOMIMETIC EFFECT OF 5-HYDROXYTRYPTAMINE ON THE ISOLATED HEART OF THE RABBIT

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1 Rabbit isolated hearts, perfused by the Langendorff technique, were used to investigate the indirect sympathomimetic effects of 5-hydroxytryptamine (5-HT). Comparisons were made with noradrenaline and with two indirectly acting sympathomimetic agents with entirely different mechanisms of action, tyramine and dimethylphenylpiperazinium (DMPP).

2 The cardiac stimulant effects of 5-HT, tyramine and DMPP were inhibited by propranolol and practolol and the pA_2 values obtained were similar to those obtained with noradrenaline as the agonist.

3 Responses to 5-HT, tyramine and DMPP were greatly reduced on hearts from rabbits pretreated with 6-hydroxydopamine. Such hearts had less than 7% of their normal catecholamine concentration and no fluorescence characteristic of noradrenaline in the cardiac sympathetic nerves could be demonstrated.

4 Rapid, reversible and selective tachyphylaxis to 5-HT was demonstrated during perfusion with 5-HT. In hearts desensitized to DMPP by perfusion with DMPP, responses to 5-HT were also reduced.

5 Perfusion of hearts with colchicine inhibited stimulant responses to 5-HT and DMPP but had little effect on responses to noradrenaline or tyramine.

6 Desmethylimipramine enhanced cardiac stimulant responses to noradrenaline and to a lesser extent, those to 5-HT and DMPP. Responses to tyramine were consistently inhibited by desmethylimipramine.

7 Tetrodotoxin abolished responses of the heart to electrical nerve stimulation but left responses to noradrenaline, 5-HT and DMPP unaffected.

8 5-HT, tyramine and DMPP evoked ^{3}H-release from hearts whose neuronal noradrenaline stores had been labelled by perfusion with [^{3}H]-(–)-noradrenaline. The pattern of release evoked by 5-HT was similar to that of DMPP but differed from that of tyramine.

9 Reducing the calcium concentration in the Tyrode solution from 3.6 to 0.2 mEq/l did not affect ^{3}H-overflow after tyramine but greatly inhibited that evoked by 5-HT and DMPP.

10 The results confirm that the stimulant effects of 5-HT on the rabbit isolated heart are the result of noradrenaline release. They further suggest that the site of the release is the terminal sympathetic nerve network. The mechanism of release shows more similarities to that of DMPP (calcium-dependent depolarization and exocytosis) than to that of tyramine (neuronal uptake and stoichiometric displacement).

Introduction

5-Hydroxytryptamine (5-HT) stimulates the rate and force of contraction of the rabbit isolated heart and the effects are abolished by reserpine suggesting mediation by catecholamine release (Jacob & Poite-Beviérre, 1960). This action has now been further investigated. In particular, 6-hydroxydopamine, which selectively destroys sympathetic nerves (Thoenen, 1972), has been used to define more precisely the source of the catecholamine released by 5-HT. Further, and in order to clarify the mechanism of its releasing action, 5-HT has been compared with two compounds, both indirect sympathomimetics, but with entirely different mechanisms of action. First, tyramine, which releases noradrenaline by stoichiometric displacement after being taken up into the nerve fibres by active transport (Trendelenburg, 1972). Second, dimethylphenylpiperazinium (DMPP), which releases noradrenaline by depolarization of the nerve terminals as a result of activation of specific receptor sites (Muscholl, 1970).

A preliminary account of some of these findings has already appeared (Fozard & Mwaluko, 1975a).

Methods

Perfusion of the heart

Rabbits of either sex weighing 1.6 to 2.8 kg were given heparin (500 u/kg) into a marginal ear vein. Two to 5 min later they were stunned by a blow to the head and bled. Hearts were rapidly removed some with the right sympathetic nerves attached (Huković & Muscholl, 1962) and perfused according to the Langendorff technique with modified Tyrode solution at 35.5°C. The Tyrode solution (concentrations in g/l: NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaHCO_3$ 1.0, NaH_2PO_4 0.05, glucose 1.0 and ascorbic acid 0.01) was gassed with a mixture of 95% O_2 and 5% CO_2. Perfusion pressure was maintained at 60 cm water. In some experiments the concentration of calcium chloride was reduced to 0.011 g/l (Lindmar, Löffelholz & Muscholl, 1967). Right ventricular tension and rate and right atrial tension were recorded as described by Fozard & Muscholl (1971).

Design of the experiments

Dose-effect curves to 5-HT, noradrenaline, tyramine and DMPP on atrial and ventricular tension development and ventricular rate were established on separate hearts by bolus injections of each compound. In general 5 min was the interval between doses, but if the effects of an earlier dose were still in evidence, this interval was extended. In most hearts, after a 15 min interval, a second dose-effect curve was obtained for a given agonist. Thus, two quantitative means of comparison were possible. First, assessment could be made of the effects of pretreatment with 6-hydroxydopamine by comparison of the initial dose-effect curves obtained on hearts from untreated and pretreated animals. Second, by including a modifying drug in the perfusion solution immediately after the first dose-effect curve had been established, a comparison could be made between the changes produced in the second dose-effect curve by the modifying drug and the effects in the control situation where no modifying drug was present.

Values for pA_2 were estimated using cardiac rate as the index of the response by the method of Arunlakshana & Schild (1959).

In order to investigate selective and cross-tachyphylaxis between the agonist drugs, hearts were set up as described above and a response to an ED_{50} bolus injection of either 5-HT (8 µg), noradrenaline (0.64 µg), tyramine (40 µg) or DMPP (40 µg) was established. In control experiments, the agonist ED_{50}'s were repeated twice with a 15 min interval between each challenge making three responses in all. In test experiments, the hearts were perfused with various concentrations of the agonist drugs immediately after the first ED_{50} response had been obtained and during the second ED_{50} injection. The perfusion fluid was changed back to control Tyrode solution immediately after the second ED_{50} response had been obtained and the third ED_{50} was injected 15 min later. Quantitative comparisons were made between the second responses in the control series and the equivalent responses obtained during perfusion with agonist drugs in the test experiments.

Experiments with [^{3}H]-(—)-noradrenaline

The endogenous cardiac neuronal noradrenaline stores were labelled with [^{3}H]-noradrenaline by the procedure described by Starke (1971). Chromatographically pure (—)-noradrenaline-[7-^{3}H] (sp. act. 7.2 Ci/mmol) was diluted freshly each day with unlabelled noradrenaline to give the working stock solution. This was infused at the rate of 0.1 ml/min (with a Palmer slow injection apparatus) into the arterial inflow to give a final concentration in the medium of 10 ng/ml and radioactive concentration of 43 nCi/ml. In these experiments the flow rate was maintained at 25 ml/min by means of a roller pump (Watson-Marlowe Type MHRE 200), and the duration of the loading period was 12 minutes.

In control experiments, the whole of the venous outflow was collected in 20 three min aliquots during a 60 min washout period. Test doses of 5-HT, tyramine and DMPP were introduced by bolus injections into the arterial inflow, at times 30, 42 and 54 min respectively after the end of the loading period. Samples of perfusate were collected at various time intervals before, during and after drug challenge as detailed in the Results section.

The total radioactivity contained in each sample of perfusate was measured by dispersing a 1 ml aliquot in 10 ml of scintillation fluid (0.1 g dimethyl POPOP, 5.5 g diphenyloxazole, 333 ml Triton X-100, 667 ml toluene) and counting in a Packard Tri-Carb Scintillation Spectrometer (Model 3320). Counting efficiency and quenching were monitored by external standardization and the results corrected accordingly.

Pretreatment with 6-hydroxydopamine

Rabbits were pretreated with 6-hydroxydopamine after the method of Fozard, Kelly & Small (1973). The degree of destruction of the cardiac sympathetic nerves was assessed by fluorimetric assay of the endogenous cardiac noradrenaline concentrations (Welch & Welch, 1969) and by histochemical fluorescence microscopy (Spriggs, Lever, Rees & Graham, 1966)..

Statistical analysis

All measurements of variations of means quoted are standard errors. Student's *t* test was used to assess the significance of differences between mean values. The number of observations is indicated by *n*.

Drugs

The drugs used were atropine sulphate (Macfarlan Smith), colchicine (Koch-Light), dimethylphenylpiperazinium iodide (Emmanuel), heparin (Evans), 5-hydroxytryptamine creatinine sulphate (Koch-Light), 6-hydroxydopamine hydrobromide (Whatman Biochemicals), (−)-noradrenaline bitartrate (Koch-Light), practolol and propranolol hydrochloride (ICI), tetrodotoxin (Sankyo), tyramine hydrochloride (Koch-Light).

Results

Responses of the rabbit heart to 5-hydroxytryptamine; use of Tyrode solution containing atropine

In untreated hearts, responses to 5-HT over the dose range used (0.5 to 512 μg) were usually biphasic consisting of an initial negative chronotropic and inotropic phase followed by a stimulant response. Responses to DMPP were similarly routinely biphasic. The initial inhibitory responses obtained with 5-HT and DMPP could be abolished by perfusion with low concentrations of atropine (Fozard & Muscholl, 1971; Mwaluko, 1975). Since this work was concerned solely with the mechanism of the stimulant response to 5-HT, all experiments have been carried out in the presence of atropine, 0.5 μg/ml. This concentration of atropine is devoid of nicotinic receptor blocking activity (Lindmar, Löffelholz & Muscholl, 1968) and can be considered to be selectively anti-muscarinic.

Effects of propranolol and practolol on cardiac responses to noradrenaline, 5-hydroxytryptamine, tyramine and dimethylphenylpiperazinium

Responses to all the agonists were inhibited by low concentrations of propranolol and practolol. The pA$_2$ values obtained for the antagonism are presented in Table 1. For each antagonist, the values obtained with 5-HT were similar to those obtained with the indirectly acting sympathomimetic amines, tyramine and DMPP. They were also similar to those obtained with noradrenaline which stimulates the β-adrenoceptors directly.

Effects of pretreatment with 6-hydroxydopamine on cardiac responses to 5-hydroxytryptamine, dimethylphenylpiperazinium, tyramine and noradrenaline

Pretreatment with 6-hydroxydopamine resulted in a fall in the cardiac noradrenaline concentration from 842 ± 112 ng/g ($n=9$) to 62.5 ± 29.0 ng/g ($n=6$) which represents a highly significant ($P<0.001$) reduction of 93% from control values. In normal atria and ventricles processed for histochemical fluorescence microscopy, the specific fluorescence attributable to noradrenaline in the cardiac sympathetic nerves could be seen. After pretreatment with 6-hydroxydopamine the fluorescence was greatly reduced and in most fields examined it was completely absent (Mwaluko, 1975).

Figure 1 shows that stimulation of cardiac rate evoked by 5-HT, tyramine and DMPP was greatly reduced in hearts from animals pretreated with 6-hydroxydopamine. In contrast, the sensitivity of these hearts to noradrenaline was somewhat increased compared to the equivalent controls.

Experiments demonstrating tachyphylaxis to 5-hydroxytryptamine and dimethylphenylpiperazinium, its reversibility and selectivity

In the control experiments shown as the upper histograms in Figure 2, responses to ED$_{50}$ doses of 5-HT, DMPP, noradrenaline and tyramine were

Table 1 pA$_2$ values (± s.e. mean) for the antagonism by propranolol and practolol of the stimulant responses on cardiac rate produced by noradrenaline, 5-hydroxytryptamine (5-HT), tyramine and dimethylphenylpiperazinium (DMPP)

	Noradrenaline	n	*5-HT*	n	*Tyramine*	n	*DMPP*	n
Propranolol	8.42 ± 0.08	8	8.43 ± 0.24	5	8.29 ± 0.12	3	8.45 ± 0.16	3
Practolol	6.04 ± 0.26	3	6.16 ± 0.14	4	6.09 ± 0.11	3	5.92 (6.31) (5.52)	2

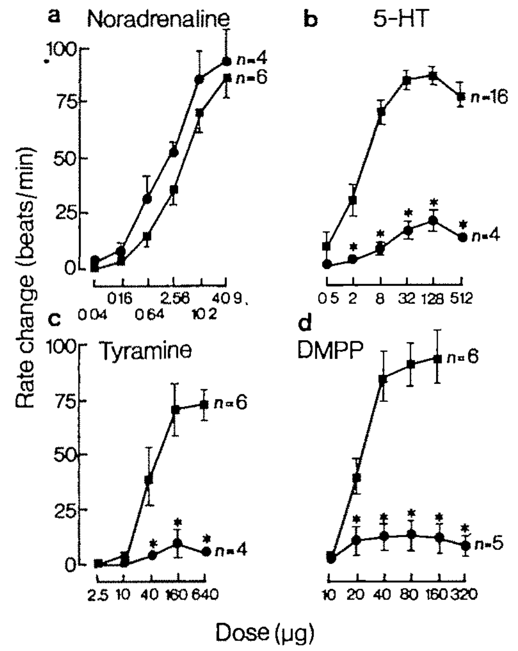

Figure 1 Effects of 6-hydroxydopamine on cardiac rate responses to bolus injections of (a) noradrenaline, (b) 5-hydroxytryptamine (5-HT), (c) tyramine and (d) dimethylphenylpiperazinium (DMPP). Atropine, 0.5 µg/ml was present throughout the experiments. (■) Hearts from untreated animals; (●) hearts from animals pretreated with 6-hydroxydopamine.

* Indicates a significant difference ($P < 0.05$) between response magnitudes at the same dose levels.

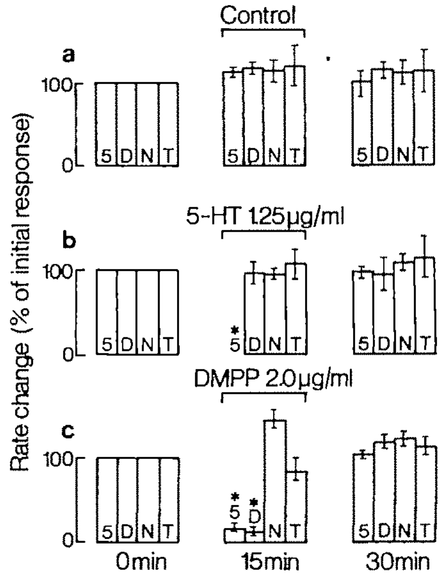

Figure 2 Tachyphylaxis and cross-tachyphylaxis during perfusion with 5-hydroxytryptamine (5-HT) and dimethylphenylpiperazinium (DMPP). Atropine, 0.5 µg/ml, was present throughout the experiments. The histograms represent the rate responses to bolus injections of ED_{50} doses of 5-HT (5), DMPP (D), noradrenaline (N) and tyramine (T) established at time zero (left hand histograms) and repeated twice at 15 min intervals. Responses are expressed as percentages of the initial rate response which was taken as 100%, and the vertical lines indicate the standard errors of the mean values calculated from 4 to 7 individual experiments. (a) Upper series: drug-free Tyrode solution throughout. (b) Middle series: 5-HT, 1.25 µg/ml present in perfusion fluid during establishment of second responses. (c) Lower series: DMPP, 2.0 µg/ml, present in perfusion fluid during establishment of second responses.

* Indicates a significant ($P < 0.05$) difference from the equivalent value in the control series.

established and repeated twice at 15 min intervals. When expressed in terms of the initial responses, no significant variation was detectable. In the test experiments, the second responses in the series were elicited during perfusion with either 5-HT, 1.25 µg/ml, or DMPP, 2.0 µg/ml, concentrations found in preliminary experiments just to abolish responses to the respective agonists. Although perfusion with each compound produced an initial sympathomimetic effect, these were not sustained and at the time of retesting the bolus injections, the baseline cardiac rates were not significantly different from those recorded immediately before the first responses in the series (Mwaluko, 1975).

During perfusion with 5-HT, 1.25 µg/ml, the responses to bolus injections of 5-HT were abolished, yet those to DMPP, noradrenaline and tyramine were little changed. Fifteen min after changing back to drug-free Tyrode solution, responses to 5-HT were back to control levels. With DMPP, 2.0 µg/ml, present in the perfusion solution, responses to bolus injections of 5-HT and DMPP were significantly reduced, those to noradrenaline were increased and those to tyramine were not significantly altered. After 15 min perfusion in drug-free Tyrode solution responses to all the agonists had returned to control levels.

Effects of colchicine on cardiac responses to 5-hydroxytryptamine, noradrenaline, tyramine and dimethylphenylpiperazinium

Figure 3 shows the effects of colchicine on the change in cardiac rate produced by bolus injections of 5-HT, noradrenaline, tyramine, and DMPP. In the control experiments in the upper part of the figure, two dose-response curves were established with a 15 min

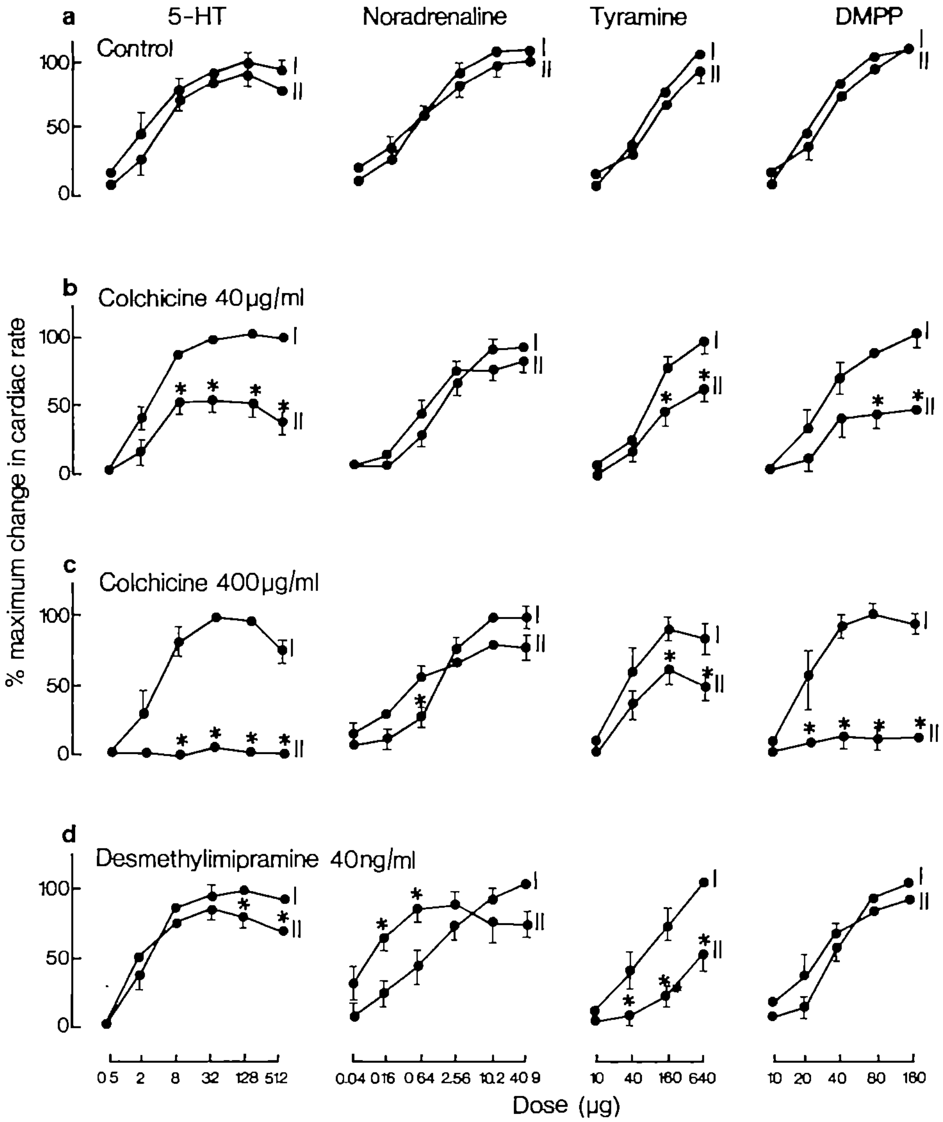

Figure 3 Effects of colchicine and desmethylimipramine on cardiac rate responses to bolus injections of 5-hydroxytryptamine (5-HT), noradrenaline, tyramine and dimethylphenylpiperazinium (DMPP). Atropine, 0.5 µg/ml was present throughout the experiments. (a) Upper graphs: control experiments, agonist dose-response curves established (I) and repeated after a 15 min interval (II). Lower graphs: (b) colchicine, 40 µg/ml and (c) 400 µg/ml, (d) desipramine, 40 ng/ml included in perfusion fluid after the first dose-response curve (I) and throughout the second dose-response curve (II). The points represent the mean values from 3 to 5 individual experiments with standard errors.

* Indicates a significant difference ($P < 0.05$) between response magnitudes at the same dose levels.

interval between them and for each agonist, responses proved to be reproducible. In the test experiments, hearts were perfused with Tyrode solution containing colchicine immediately after the first dose-response curve had been established. Colchicine, 40 and 400 µg/ml produced a concentration-dependent inhibition of responses to 5-HT and DMPP. In each case, the antagonism could not be surmounted by increasing the doses of either 5-HT or DMPP. In contrast, stimulation of cardiac rate evoked by noradrenaline and tyramine was not inhibited by colchicine in a concentration-dependent fashion, although as Figure 3 shows, small but significant depression of the responses to tyramine was observed at each concentration of colchicine used.

Effects of desmethylimipramine on the cardiac responses to 5-hydroxytryptamine, noradrenaline, tyramine and dimethylphenylpiperazinium

The experimental design of these experiments was identical to that described for colchicine outlined above. In the presence of 40 ng/ml desmethylimipramine, the stimulant effects of 5-HT and DMPP were modified similarly. First, the magnitudes of the responses were little changed compared to control values (Figure 3). Second, in each case there was a considerable prolongation of the duration of the cardiac rate response. Responses to noradrenaline were clearly enhanced during perfusion of the hearts with desmethylimipramine (Figure 3) and as with 5-HT and DMPP, the duration of the responses was markedly prolonged. In direct contrast, the cardiac stimulant response to tyramine was inhibited over the whole dose-range by desmethylimipramine (Figure 3).

Effects of tetrodotoxin on cardiac responses evoked by electrical stimulation of the cardiac sympathetic nerves and by bolus injections of noradrenaline, 5-hydroxytryptamine and dimethylphenylpiperazinium

Electrical stimulation of the cardiac sympathetic nerves by the method of Huković & Muscholl (1962) evoked frequency-dependent increases in ventricular tension development and ventricular rate. Tetrodotoxin, 0.5 µg/ml, abolished responses to electrical nerve stimulation but did not affect the response to a bolus injection of noradrenaline (Mwaluko, 1975). Responses to 5-HT and DMPP were completely unaffected by perfusion with tetrodotoxin (Figure 4).

Drug-evoked release of ^3H from hearts whose noradrenaline stores had been labelled by perfusion with [^3H]-(−)-noradrenaline; effects of lowered extracellular Ca^{2+} concentration

The cardiac neuronal noradrenaline stores were labelled by perfusion with [^{3}H]-noradrenaline (10 ng/ml and 43 nCi/ml) for 12 min (Starke, 1971). In contrast to tyramine and DMPP, bolus injection of a dose of 5-HT representing an ED_{50} on mechanical performance (8 µg) proved only weakly effective as a stimulant of ^{3}H-release from the heart. This observation, which may reflect a restricted locus of transmitter releasing action of 5-HT (Mwaluko, 1975), is being further investigated. However, in order to make quantitative comparisons in the present experiments, a higher dose of 5-HT (512 µg) was chosen to stimulate ^{3}H-release. Qualitatively identical results to those presented below have been obtained in separate experiments with lower doses of 5-HT.

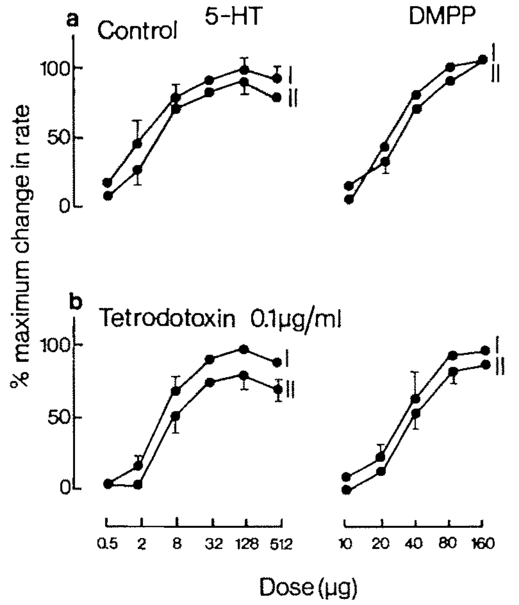

Figure 4 Effects of tetrodotoxin on cardiac rate responses to bolus injections of 5-hydroxytryptamine (5-HT) and dimethylphenylpiperazinium (DMPP). Atropine, 0.5 µg/ml was present throughout the experiments. (a) Control experiments, agonist dose-response curves established (I) and repeated after a 15 min interval (II). (b) Tetrodotoxin, 0.1 µg/ml included in the perfusion fluid after the first dose-response curve (I) and throughout the second dose-response curve (II). Other details as in Figure 3.

In control experiments, where the total venous outflow was collected over a 60 min period, there was an initial sharp fall in the rate of ^{3}H-washout during the first 10 to 12 min, which was followed by a slow but constant decline over the remaining perfusion period (Mwaluko, 1975). 5-HT (512 µg), tyramine (40 µg) and DMPP (40 µg) were administered during this period as bolus injections, 30, 42 and 54 min after the end of the loading period respectively. The venous outflow was sampled as follows: 1 min before and 1 min after the injection of agonist, the outflow was collected in 10 s aliquots. For a 2 min period before and after the 10 s collection period, 1 min samples were collected for assay. At all remaining times, venous outflow was collected in 3 min samples for radioassay.

The results are expressed as nCi/10 s in Figure 5a and nCi/min in Figure 5b. 5-HT (512 µg) evoked a marked overflow of ^{3}H into the perfusion fluid. The peak of this overflow was reached within 10 to 30 s of

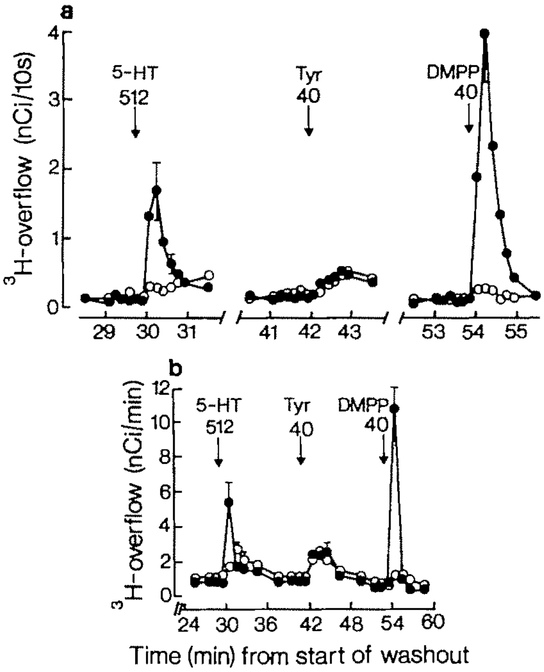

Figure 5 Spontaneous and evoked ^{3}H-overflow from hearts whose neuronal transmitter stores had been labelled by perfusion with [^{3}H]-(−)-noradrenaline (10 ng/ml, 43 nCi/ml) for 12 minutes. Atropine 0.5 µg/ml was present throughout the experiments. Zero time marks the end of the loading period with [^{3}H]-noradrenaline. Washout was with either Tyrode solution containing its normal calcium ion concentration (●), or Tyrode solution containing 6% of its normal calcium ion concentration (○). The points represent the means of 4 observations; vertical lines show s.e. mean. (a) Effect of bolus injections of 5-HT (512 µg), tyramine 40 µg (Tyr), and DMPP (40 µg), results presented as nCi/10 s. (b) Results presented as nCi/minute.

the injection and little release was evident after 1 to 1.5 min (Figure 5a). However, there was a secondary, low level, increase in ^{3}H-overflow, apparent 3 to 5 min after the injection which can be seen when the data are expressed as nCi/min (Figure 5b). DMPP (40 µg) also evoked a marked increase in ^{3}H-overflow and the pattern of the release was similar to that of 5-HT. Thus, the peak effect was reached between 10 and 30 s after administration and the ^{3}H concentration was close to pre-injection levels after 1 to 1.5 min (Figures 5a and b). Tyramine (40 µg) also caused a significant increase in ^{3}H-overflow although, in contrast to the pattern of release seen with 5-HT and DMPP, the peak of ^{3}H-overflow was not reached until 50 to 60 s after the injection (Figure 5a), and the overflow was still elevated 3 to 5 min after the injection (Figure 5b).

When the calcium ion concentration of the Tyrode solution was lowered from 3.6 to 0.2 mEq/l immediately after the loading period, the heart stopped beating and remained quiescent for the duration of the perfusion. The initial peak of ^{3}H-overflow evoked both by 5-HT and DMPP was greatly and significantly reduced by lowering the calcium ion concentration (Figures 5a and b). In contrast, lowering the extracellular calcium ion concentration had no significant effect on the ^{3}H-overflow evoked by tyramine. It was also apparent that the secondary component of the ^{3}H-release evoked by 5-HT persisted under conditions of lowered calcium ion concentration (Figure 5b).

Discussion

There are several pieces of evidence suggesting that the stimulant action of 5-HT on rabbit isolated hearts or atria is both sympathomimetic and indirect. For instance, the prototype β-adrenoceptor antagonist drug, dichloroisoprenaline, was used by Trendelenburg (1960) to antagonize successfully the stimulant response to 5-HT on rabbit atria. Further, reserpine pretreatment was used by both Jacob & Poite-Bevierre (1960) and Trendelenburg (1960) to reduce effectively the cardiac stimulant response to 5-HT. The present data extend these earlier observations by using the newer, more selective β-adrenoceptor antagonists, propranolol and practolol, by adopting a more quantitative approach to the assessment of antagonism and by using a catecholamine depleting agent, 6-hydroxydopamine, whose action is selective for the sympathetic nerves.

Thus the fact that the pA_2 values for the antagonism by propranolol and practolol of the cardiac stimulant effects of 5-HT, noradrenaline, tyramine and DMPP are similar to each other and to the values found by other workers for the antagonism of cardiac β-adrenoceptors by the two drugs (Furchgott, 1972), is convincing evidence that stimulant responses to all these drugs are the result of activation of β-adrenoceptors. Further, the mode of action of 5-HT, tyramine and DMPP can be described as indirect, the result of catecholamine release from the cardiac sympathetic nerves, since their effects were reduced by pretreatment with 6-hydroxydopamine which, unlike reserpine, does not deplete amines from chromaffin tissue (Thoenen, Tranzer & Haeusler, 1970). This latter conclusion is further supported by the observations presented in Figure 5 where 5-HT along with tyramine and DMPP evoked a release of ^{3}H from hearts whose neuronal noradrenaline stores had been labelled by prior perfusion with [^{3}H]-(−)-noradrenaline (Starke, 1971).

When explanations are sought for the mechanism of the indirect sympathomimetic actions of 5-HT, there are two principal possibilities. First, there is the well known excitatory effect of 5-HT on both parasympathetic (Rocha e Silva, Valle & Picarelli, 1953; Gaddum & Picarelli, 1957) and sympathetic (Trendelenburg, 1956; Haefely, 1974b) nervous tissue. Detailed analysis of the phenomenon using isolated sympathetic ganglia suggests that transmitter release results from depolarization of the nerve cells as a result of activation of receptors specific for tryptamines (Haefely, 1974b). With the exception of the receptor sites involved, this action is analogous to that of stimulants at nicotinic receptors such as DMPP (Haefely, 1974a;b). The other possibility which may be referred to as 'tyramine-like' is supported by the observations that 5-HT is accumulated by cardiac sympathetic nerves by active transport (Burgen & Iversen, 1965; Fillion, Lluch & Uvnas, 1971; Jester & Horst, 1972), and that 5-HT can displace endogenous noradrenaline from its storage sites within such nerves (Andeń, 1964; Gillis, 1964). Such a mechanism is claimed to operate in the indirect sympathomimetic response to 5-HT which has been demonstrated in the cat isolated spleen strip (Innes, 1962; Pluchino, 1972), nictitating membrane (Pluchino, 1972) and rat anococcygeous muscle (McGrath, 1973). The evidence from the present work allows the firm conclusion to be drawn that the indirect sympathomimetic response to 5-HT in rabbit hearts is, like DMPP, the result of neuronal depolarization and not, like tyramine, dependent on neuronal uptake and stoichiometric displacement of the stored transmitter.

First, complete and reversible tachyphylaxis to bolus injections of 5-HT was readily produced by perfusion with 1.25 μg/ml 5-HT (Figure 2). In this respect the stimulant effect of 5-HT on cardiac sympathetic nerves is similar to its actions on the parasympathetic neurones of the guinea-pig ileum (Gaddum & Picarelli, 1957) and the cells of the superior cervical ganglion of the cat (Trendelenburg, 1956; Haefely, 1974a). Tachyphylaxis to 5-HT was selective in that it was not extended to DMPP or tyramine (Figure 2), which suggests its site of action is not the nicotinic receptor and that 5-HT and tyramine have basically different mechanisms of action. In contrast, during perfusion with DMPP, 2 μg/ml, responses to both DMPP and 5-HT were considerably and similarly reduced, although those to tyramine were still very much in evidence. The possibility of an interaction at the level of the nicotinic receptors as an explanation of the phenomenon can be discounted; first, because of the selectivity of tachyphylaxis to 5-HT, and secondly because hexamethonium, in concentrations that abolish responses to DMPP, leaves responses to 5-HT unaffected (Fozard & Mwaluko, 1975b). The explanation might however be related to the non depolarizing 'adrenergic neurone blocking activity' of DMPP described for the rabbit heart by Löffelholz (1970a; b). It is easy to envisage that such a property, which is demonstrably able to inhibit the powerful depolarizing stimulus arising from electrical nerve stimulation, would also inhibit a relatively weak, chemical stimulus effected by 5-HT. However, perhaps the most important point in this context is not to establish the exact mechanism of the inhibition produced by DMPP but to highlight the differential effects of DMPP in inhibiting 5-HT more readily than tyramine. This fact once again links the mechanism of action of 5-HT with that of DMPP rather than with that of tyramine.

The use of colchicine further amplifies the association of the mechanism of action of 5-HT with that of DMPP rather than tyramine. Colchicine interferes with the exocytotic secretion of many hormones (Lacy, Howell, Young & Fink, 1968; Kraicer & Milligan, 1971; Douglas & Sorimachi, 1972; Sorimachi, Oesch & Thoenen, 1973) possibly by causing disintegration of microtubules (Borisy & Taylor, 1967). In the present experiments, responses to 5-HT and DMPP, but not those to noradrenaline or tyramine, were inhibited by colchicine (Figure 3). In the case of nicotinic agonists colchicine has been specifically suggested to interfere with some step(s) in an excitation-secretion coupling mechanism which is calcium ion-dependent and which results in the release of noradrenaline, probably by exocytosis from the noradrenergic nerve terminals (Smith & Winkler, 1972; Sorimachi *et al.*, 1973). The present results suggest a similar conclusion would be justified with 5-HT as the stimulus to release, especially as responses to tyramine, whose action is independent of both extracellular calcium ion and the process of exocytosis (Lindmar *et al.*, 1967; Thoenen, Huerlimann & Haefely, 1969; Chubb, De Potter & De Schaepdryver, 1972; Thoa, Wooten, Axelrod & Kopin, 1975) remained largely unaffected by perfusion with colchicine (Figure 3).

The reasoning behind the experiments with desmethylimipramine was that if 5-HT were releasing noradrenaline by a 'tyramine-like' mechanism then its action should be inhibited by drugs which interfere with the neuronal monoamine uptake pathway. Desipramine (40 ng/ml) has previously been shown to block noradrenaline uptake in the rabbit isolated heart (Wennmalm, 1971; Barth, Manns & Muscholl, 1975) and in the present experiments, potentiation of noradrenaline coupled with inhibition of tyramine (Figure 3) are a predictable consequence of such an action (Trendelenburg, 1972). In practice, responses to 5-HT, like those to DMPP, were not inhibited by perfusion with desipramine (Figure 3) and their durations were markedly prolonged. Thus, transmitter release evoked both by 5-HT and DMPP appears to take place independently of the neuronal amine transport mechanism. However, inhibition of reuptake of the released transmitter would be a significant factor contributing to the enhanced responses observed.

The results obtained in the experiments where endogenous neuronal noradrenaline stores were labelled with [^{3}H]-noradrenaline add further weight to the argument that the mechanism by which 5-HT stimulates the cardiac sympathetic nerves is more similar to DMPP than to tyramine. Thus, whereas 5-HT mimicked DMPP in causing a sharp peak of ^{3}H-release which was virtually complete within 1 min, tyramine showed a slow onset and decline of release with the rate of ^{3}H-overflow, expressed as nCi/min, being constant over the 3 min period immediately following the injection (Figure 5). These characteristic time courses cannot have as their bases the particular sequence of agonist injections employed in Figure 5, since identical release patterns were observed in separate preliminary experiments where the agonists were injected either alone or in entirely random sequences. DMPP has been shown to cause an 'explosive' release of noradrenaline from cardiac sympathetic nerves by a brief depolarization of the neuronal membrane which is terminated rapidly by 'autoinhibition' (Haeusler, Thoenen, Haefely & Huerlimann, 1968; Löffelholz, 1970a; b). The present data support this concept for DMPP and suggest that a similar mechanism may operate with 5-HT. Tyramine, on the other hand, must first be taken up by the neuronal membrane (Commarato, Brody & McNeill, 1969), then by the storage vesicles (Musacchio, Kopin & Weise, 1965), before displacing the stored transmitter. The relatively slow increase in the perfusate ^{3}H concentration presumably reflects these movements. Similarly, since termination of the releasing action of tyramine depends on enzymatic metabolism (Musacchio *et al.*, 1965; Smith, 1966), the decline of its releasing effect will be a more gradual phenomenon than, say, the decline of the releasing action of DMPP brought about by autoinhibition.

When the calcium concentration of the perfusion fluid was reduced to 6% of normal, the initial peak of ^{3}H-release caused by DMPP and 5-HT was abolished whereas ^{3}H-release by tyramine remained unaffected (Figure 5). These results confirm previous reports that sympathetic transmitter release evoked by DMPP is calcium-dependent (Lindmar *et al.*, 1967; Haeusler *et al.*, 1969; Chubb *et al.*, 1972; Thoa *et al.*, 1975). The demonstration that release by 5-HT is, like DMPP, dependent on extracellular calcium ion has its counterpart in the perfused cat adrenal gland, where catecholamine release evoked both by nicotinic agonists and 5-HT was abolished by omission of calcium from the perfusion medium (Douglas & Rubin, 1963; Poisner & Douglas, 1966). The results clearly distinguish between the mechanisms of action of 5-HT and tyramine and emphasize again the basic similarity between the mechanisms of action of 5-HT and DMPP.

The experiments with calcium-depleted Tyrode solution do, however, disclose that the small secondary peak of ^{3}H-overflow normally observed 2 to 5 min after the injection of 5-HT persists unchanged during calcium deprivation (Figure 5b). The simplest explanation for the observation is that at the high dose used to elicit ^{3}H-release, 5-HT has properties characteristic of DMPP (the initial peak) and tyramine (the secondary release). Such a conclusion might have been anticipated from the literature where, at high doses, 5-HT has been shown to be taken up by the sympathetic nerves and to release endogenous noradrenaline from its vesicular storage sites (see above). The possibility that the 'tyramine-like' action of 5-HT is a complicating factor in the interpretation of the data obtained using end organ response measurements can be discounted; first, because the time course of the response on mechanical performance and the 'tyramine-like' release (Figure 5b) are quite different; second, because the phenomenon is not observed with doses of 5-HT less than 512 µg, which is 64 times the ED_{50} for stimulation of mechanical performance (Mwaluko, 1975) and finally, because a 'tyramine-like' release mechanism would be incompatible with the data obtained with colchicine (Figure 3), desmethylimipramine (Figure 3) and the demonstration of selective tachyphylaxis to 5-HT (Figure 2).

Tetrodotoxin blocks propagated action potentials in nerve and muscle by preventing the rapid inward sodium current (Kuryama, Osa & Toida, 1966) and, predictably, the effects of electrical nerve stimulation were abolished during perfusion with the drug. In contrast, responses to 5-HT and DMPP remained unaltered (Figure 4). Sympathetic transmitter release by nicotinic agonists has previously been shown to be resistant to tetrodotoxin, despite action potential generation in the nerves being abolished (Haeusler *et al.*, 1968; Krauss, Carpenter & Kopin, 1970; Su & Bevan, 1970; Westfall & Brasted, 1972; Thoa *et al.*, 1975). Krauss *et al.* (1970) described the mechanism of action of nicotinic agonists as resulting from propagated depolarization and an increased calcium permeability of the nerve terminal that is independent of a propagated action potential. The present results suggest a similar description would be appropriate to the action of 5-HT.

In conclusion, the mechanism of the indirect sympathomimetic effect of 5-HT on the rabbit isolated heart has been shown to be fundamentally different from that of tyramine. In only one respect, however, has it been shown to differ from that of DMPP, and that is in the nature of the sites which trigger the response (Figure 3). By analogy with data from the superior cervical ganglion where receptor sites specific for tryptamines appear to exist on the cell bodies of the sympathetic neurones (Haefely, 1974b), the likelihood is that transmitter release by 5-HT is mediated through similar sites on the terminal sympathetic fibres. To establish this as a fact is the subject of our continuing investigations (Fozard & Mwaluko, 1975b).

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THE DIFFERENT EFFECTS OF D-600 (METHOXYVERAPAMIL) ON THE RELEASE OF ADRENAL CATECHOLAMINES INDUCED BY ACETYLCHOLINE, HIGH POTASSIUM OR SODIUM DEPRIVATION

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1 Bovine adrenal glands were perfused with Locke solution and catecholamine release was induced by acetylcholine, by a depolarizing concentration of potassium, or by omission of sodium from the perfusion fluid.

2 D-600 (methoxyverapamil) at the concentration of 30 µM produced a 23% inhibition of catecholamine release evoked by acetylcholine (0.1 mM) in the presence of physostigmine (10 µM).

3 A concentration of D-600 of 0.3 mM produced 86% and 85% inhibition in the output of catecholamines in response to acetylcholine and high potassium respectively.

4 D-600 (0.3 mM) failed to block the release of catecholamines evoked by sodium deprivation.

5 The results suggest the involvement of intracellular calcium in the exocytotic release of catecholamines induced by sodium omission.

Introduction

When the adrenal medulla is stimulated by either acetylcholine or depolarizing concentrations of K^+, catecholamines and other soluble components of the chromaffin granules are released to the cell exterior by a process of exocytosis (Smith & Winkler, 1972). The release of catecholamines induced by the above stimuli requires the presence of extracellular Ca^{2+} (Douglas, 1968). In contrast to the above observations, we have recently shown that the omission of Na^+ from the extracellular environment evokes release of catecholamines by exocytosis in the absence of extracellular Ca^{2+} (Lastowecka & Trifaró, 1974).

Methoxyverapamil (D-600) is a drug that blocks both calcium influx in excitable tissues (Fleckenstein, 1971; Fleckenstein, Grun, Tritthart & Byon, 1971; Mayer, van Breemen & Casteels, 1972; Baker, Meves & Ridgway, 1973) and hormone release from the neurohypophysis (Dreifuss, Grau & Nordmann, 1973; Russell & Thorn, 1974). Therefore, the present study was undertaken to examine the effect of D-600 on the release of catecholamines evoked by all of the above stimuli.

Methods

Bovine adrenal glands obtained from a local slaughterhouse were perfused and stimulated *in vitro* as described by Trifaró, Poisner & Douglas (1967).

Perfusion fluids

(a) The main perfusion fluid was phosphate-buffered-Locke solution of the following composition (mM): NaCl 154, $CaCl_2$ 2.2, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85 and dextrose 10, Na^+-free Locke solution was of the same composition as the main perfusion fluid except that NaCl was replaced by an osmotically equivalent concentration of sucrose; (c) high potassium Locke solution contained 56 mM K^+ of which 53 mM was as KCl and 3 mM as K_2HOP_4 and KH_2PO_4. In this solution NaCl was reduced by an equivalent amount (50.4 mM). All the above solutions contained 0.06% ethanol. This was the final concentration of ethanol required in the experiments with D-600. This concentration of ethanol did not affect either the spontaneous or the evoked release of catecholamines. All solutions were equilibrated with 5% CO_2 in O_2, and the final pH of the solutions was 7.2. The glands were perfused at room temperature (25°C) by means of a multichannel peristaltic pump (Büchler) at a constant rate of flow of 10 ml/minute.

Samples of the perfusate were collected at 1 min intervals in ice-chilled tubes containing 10 µl of 1.9 N

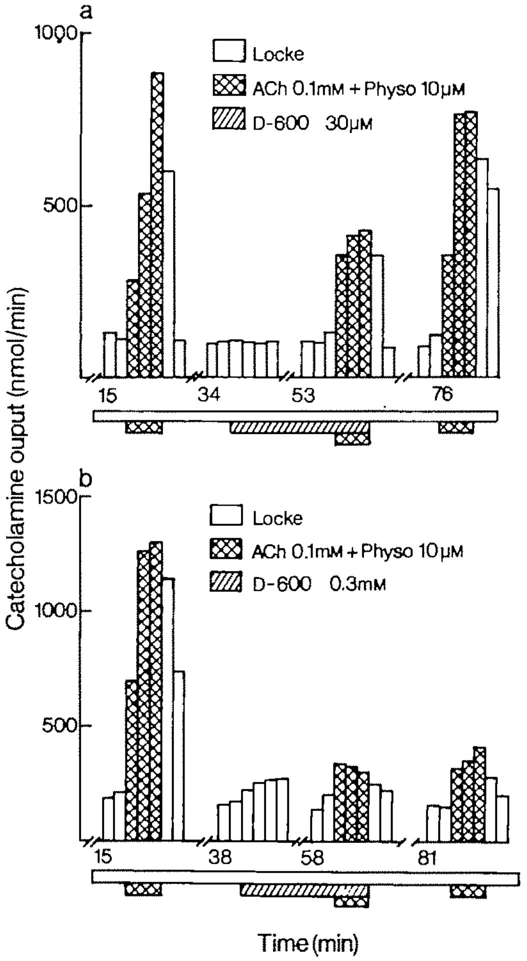

Figure 1 Effect of D-600 on acetylcholine-evoked release of catecholamines. In this, and in all subsequent figures, the graphs show the rate of catecholamine output (nmol/min) from perfused bovine adrenal glands. (a) and (b) show the stimulant effect of acetylcholine (0.1 mM) in the presence of physostigmine (Physo, 10 μM) (cross-hatched areas), during perfusion with Locke solution (open areas) or Locke solution containing D-600 at the concentration of 30 μM (diagonally hatched areas) in (a) and 0.3 mM (diagonally hatched areas) in (b). Glands were perfused at room temperature (25°C) with a flow rate of 10 ml/minute. The perfusing solutions were gassed with a mixture of 5% CO_2 in O_2. Samples were collected from the perfusates at 1 min intervals; these were assayed for catecholamine content as indicated in the methods section.

HCl per ml perfusate. The catecholamine content of the perfusates was determined by the trihydroxyindole fluorometric method (Anton & Sayre, 1962).

Chemicals

The chemicals were obtained from the following sources: physostigmine (eserine) sulphate, Sigma Chemical Company; acetylcholine chloride, Welcker Laboratories; and sucrose (density gradient grade), Schwarz-Mawn. Methoxyverapamil (D-600) was a generous gift from Drs Oberdorf and Sharma, Knoll A.C., Ludwigshafen.

Results

Seven adrenal glands were perfused for three successive periods of 3 min each with Locke solution containing acetylcholine (0.1 mM) and physostigmine (10 μM). Each of these 3 min stimulation periods was separated from the next by 20–25 min perfusion with normal Locke solution. When compared to the first acetylcholine stimulation, catecholamine outputs during the second and third stimulation periods decreased by 10–20 and 20–30% respectively. This decrease in the adrenal medullary responses to successive exposures to secretagogues has been reported previously (Douglas & Rubin, 1961; Lastowecka & Trifaró, 1974). Seven other adrenal glands were also perfused and stimulated as indicated above, but during the second acetylcholine stimulation, D-600 (0.3 mM) was present in the perfusion fluid. D-600 was introduced into the medium 20 min before the second acetylcholine stimulation and it was withdrawn from the perfusion fluid together with the acetylcholine (Figure 1). Perfusion of the adrenals with normal Locke solution containing 0.3 mM D-600 did not produce any significant change in the spontaneous release of catecholamines. Figure 1b shows that the response to acetylcholine was almost completely blocked. Furthermore, it should also be noticed that 20 min after the removal of D-600 from the perfusion medium, the catecholamine output in response to acetylcholine stimulation did not reach the expected values (Figure 1b). Table 1 shows outputs of catecholamines in response to acetylcholine stimulations performed before and during the exposure to D-600. Under these conditions, that is, in the presence of D-600, the catecholamine outputs in response to acetylcholine stimulation were 0.9–17.2% of that obtained during the preceding stimulations in the absence of D-600. This inhibition of the acetylcholine-evoked release of catecholamines was highly significant, as indicated in Table 2. When the concentration of D-600 was one order of magnitude smaller (30 μM) the inhibition of the catecholamine release induced by acetylcholine was 23% ($n = 3$) (Figure 1a). Moreover, in contrast to the results obtained in the presence of 0.3 mM D-600 (Figure 1b), the response to the subsequent acetylcholine stimulation completely recovered (Figure 1a).

As in the case of acetylcholine stimulation, the adrenal medullary responses to depolarizing concentrations of K^+ (56 mM) were blocked by D-600. Figure 2 shows that the release of catecholamines in response to 56 mM K^+ was inhibited by D-600 (0.3 mM), and as during acetylcholine stimulation, the responses to high K^+ did not recover until 60 min after the removal of D-600 from the perfusion medium. The degree of inhibition produced by D-600 during stimulation either by acetylcholine or by high K^+ was similar (Table 2).

In contrast to the above observations, D-600 failed to inhibit the release of catecholamines produced by the omission of Na^+ from the extra-cellular medium (Figure 3). The values of the output of catecholamines obtained during Na^+-deprivation, either in the presence or in the absence of D-600, are shown in Table 1. As Table 2 also indicates, D-600 produced no

Table 1 Effect of D-600 on the output of catecholamines (CA) evoked either by acetylcholine (ACh 0.1 mM) or by Na^+ omission. Adrenal glands were perfused during the second stimulation period with Locke solution containing D-600 (0.3 mM)

		Increase in catecholamine release (nmol/min)		
Expt. No.	*Condition*	*1st Stimulation*	*2nd Stimulation + D-600*	*CA released during 2nd stimn. as % of release during 1st stimn. (= 100%)*
1	ACh Locke	+ 305	+ 43	14.1
2	ACh Locke	+ 408	+ 70	17.2
3	ACh Locke	+ 511	+ 71	13.9
4	ACh Locke	+ 565	+ 5	0.9
5	ACh Locke	+ 682	+ 30	4.4
6	ACh Locke	+ 767	+ 75	9.8
7	ACh Locke	+ 1079	+ 157	14.6
8	Na^+-free Locke	+ 842	+ 792	94.1
9	Na^+-free Locke	+ 876	+ 933	106.5
10	Na^+-free Locke	+ 939	+ 848	90.3
11	Na^+-free Locke	+ 1428	+ 780	68.6

Table 2 Effect of D-600 on the release of catecholamines (CA) from the adrenal medulla. Adrenal glands were perfused with Locke solution and stimulated in the presence or in the absence of D-600 (0.3 mM) by acetylcholine (0.1 mM) in the presence of physostigmine (10 μM), by 56 mM KCl and by Na^+ deprivation

Condition		
1st Stimulation	*2nd Stimulation*	*CA released during 2nd stimn. as % of release during 1st stimn. (= 100%)*
ACh	ACh	84.5 ± 1.2[*] ($n = 7$)
ACh	ACh + D-600	10.7 ± 2.3[†] ($n = 7$)
K^+	K^+	91.9 ± 2.2 ($n = 4$)
K^+	K^+ + D-600	13.6 ± 4.5[†] ($n = 3$)
Na^+-free	Na^+-free	91.1 ± 11.1 ($n = 13$)
Na^+-free	Na^+-free + D-600	89.8 ± 7.9 ($n = 4$)

[*] Mean ± s.e. mean; n = number of tests.

[†] $P < 0.001$

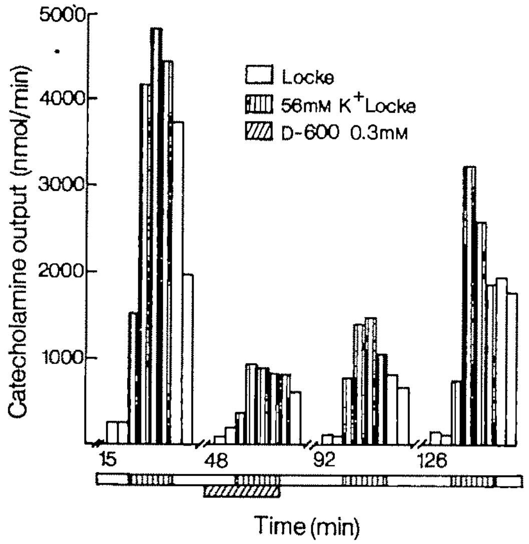

Figure 2 Effect of D-600 on the output of catecholamines induced by a depolarizing concentration of KCl. A bovine adrenal gland was perfused for 17 min with Locke solution (open areas) and was subjected to four periods of stimulation by 56 mM K^+ Locke solution (vertically hatched areas). Twenty min before the second stimulation, D-600 (0.3 mM) was added to the perfusion fluid (diagonal hatching). D-600 was removed from the medium at the end of the second stimulation period. Similar results were obtained with two other glands. Other conditions were as described in Figure 1.

significant changes in the catecholamine output induced by Na^+ omission.

In order to appreciate better the contrasting effects of D-600 on the release of catecholamines induced by either acetylcholine or by Na^+-deprivation, a series of combined experiments were also carried out. One of these experiments is depicted in Figure 4, which clearly shows that D-600 (0.3 mM) blocked acetylcholine-evoked amine release without affecting the release induced by Na^+ omission. Furthermore, the catecholamine output in response to the acetylcholine stimulation which followed the stimulation by Na^+-deprivation, was still depressed and only reached normal values of output during the subsequent acetylcholine stimulation. This stimulation was applied approximately 60 min after the removal of D-600 from the perfusion medium (Figure 4).

Discussion

The requirement of extracellular Ca^{2+} for the release of catecholamines from the adrenal medulla induced by acetylcholine or by depolarizing concentrations of K^+ is well established (Douglas, 1968; Smith & Winkler, 1972). Furthermore, the blocking effect of Mg^{2+} in the release process of the adrenal medulla is also well documented (Douglas, 1968; Smith & Winkler, 1972). Similar results on the need of extracellular Ca^{2+} and on the blocking effect of Mg^{2+} have been obtained for other secretory tissues (Rubin, 1974). Although it is still not clear where calcium ions are required in the secretory process, there is a suggestion that under physiological conditions, Ca^{2+} entry might be the first step in stimulus-secretion coupling. However, under certain experimental conditions, as for example during Na^+-deprivation, it is possible to induce release of catecholamines from the adrenal medulla during perfusion with solutions devoid of Ca^{2+} (Lastowecka & Trifaró, 1974). Since Na^+-deprivation, like acetylcholine, induces release by exocytosis, calcium ions might be involved in this process (Lastowecka & Trifaró, 1974). These calcium ions could originate from either intracellular, or perhaps even extracellular sources, since it is difficult to produce a state in which the tissue is free of extracellular Ca^{2+} because some Ca^{2+} may leak from adjacent tissue structures (Thorn, 1974). This holds for perfusion experiments with calcium-free medium, even in the presence of chelating agents

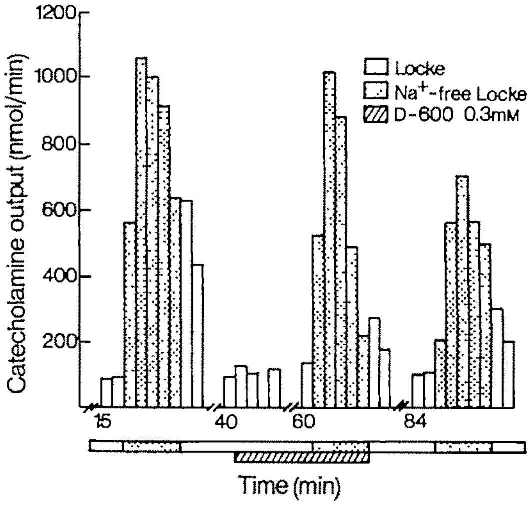

Figure 3 The lack of effect of D-600 on the release of catecholamines produced by the omission of Na^+ from the perfusion fluid. A bovine adrenal gland was perfused alternately with Locke solution (open areas) and Na^+-free Locke solution (stippled areas). During the second perfusion period with Na^+-free Locke solution, 0.3 mM D-600 (diagonally hatched areas) was present in the fluid. Similar results were obtained in three other experiments. Other conditions were as described in Figure 1.

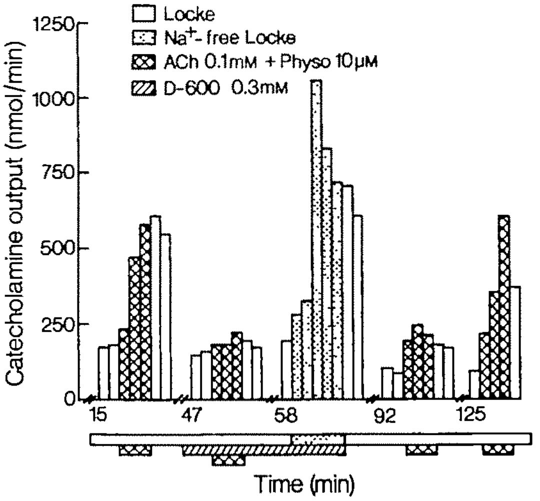

Figure 4 Contrasting effects of D-600 on the release of catecholamines induced by acetylcholine and by Na$^+$-deprivation. A bovine adrenal gland was perfused with Locke solution (open areas) and was subjected to four periods of stimulation by acetylcholine (cross hatched areas). Twenty min before the second acetylcholine stimulation 0.3 mM D-600 (diagonally hatched areas) was added to the perfusion fluid. During the last 5 min of perfusion with Locke solution containing D-600, the NaCl of the medium was replaced by an osmotic equivalent amount of sucrose (stippled areas). Similar results were obtained in three other experiments. Other conditions were as described in Figure 1.

(EDTA or EGTA). Although the amount of Ca^{2+} may be small, it could be large enough to enter the cell and trigger catecholamine release, especially in a condition such as Na$^+$-deprivation. Experiments carried out in other excitable tissues, as for example the squid axon and the heart muscle, have shown that, during Na$^+$ omission from the extracellular environment, there is an increased Ca^{2+} entry (Baker & Reuter, 1975). Because of all of the above reasons, it was thought that methoxyverapamil (D-600) might be a useful tool in order to obtain more information on the role of calcium ions in the release of catecholamines evoked by different agents and conditions. D-600 has been shown to inhibit both the release of vasopressin and oxytocin from the neurohypophysis (Dreifuss *et al.*, 1973; Russell & Thorn, 1974; Thorn, 1974). In addition, D-600 blocks the ^{45}Ca uptake by the neurohypophysis (Dreifuss *et al.*, 1973), the transmembrane slow calcium current in cardiac fibres (Kohlhardt, Baüer, Krause & Fleckenstein, 1972) and the influx of calcium through the 'late calcium channel' in the squid axon (Baker *et al.*, 1973; Baker & Reuter, 1975).

Our results provide new evidence on the blocking effect of D-600 on another endocrine tissue. Perfusion of adrenal glands with D-600 at a concentration of 0.3 mM produced a 85–86% inhibition of the responses to both acetylcholine and high K$^+$ stimulation. The concentration of D-600 required to produce a blockage in our experiments was somewhat greater than that necessary to block the responses in other tissues: 0.19 mM D-600 blocked calcium entry in the squid axon (Baker *et al.*, 1973); and D-600 concentrations of 20 μM and 50 μM inhibited the release of hormones from the neurohypophysis in response to depolarizing concentrations of potassium and to electrical stimulation respectively (Dreifuss *et al.*, 1972; Russell & Thorn, 1974). The lower sensitivity of the adrenal medullary tissue to D-600 may be due either to a low affinity of D-600 for the chromaffin cell or to a high density of calcium channels in this tissue. If this latter possibility were the case, it would provide an explanation for the discrepancy between the findings in the neurohypophysis and in the adrenal medulla. A very small amount of calcium seems to be required for the release of hormone in the neurohypophysis (Russell & Thorn, 1974; Thorn, 1974), whereas release of catecholamines from the adrenal medulla is accompanied by a large uptake of calcium (Douglas & Poisner, 1962).

In spite of the differences between the optimal blocking concentrations of D-600 in the neurohypophysis (Russell & Thorn, 1974; Thorn, 1974) and in the adrenal medulla, the slow reversibility (about 60 min) of the blocking effect of the drug was similar in both tissues.

Since Ca^{2+} entry through the late calcium channel of the squid axon is blocked by the same agents (i.e., D-600, Mg^{2+}, Mn^{2+}, etc.) which block secretion, Baker *et al.* (1973) have suggested that Ca^{2+} may enter the secretory cells through a channel of properties similar to those of the late calcium channel of the squid axon. The present results, showing that D-600 blocks both acetylcholine and high K$^+$-induced release of catecholamines, are in agreement with Baker's suggestion. The failure of D-600 to block the release of catecholamines in response to Na$^+$-deprivation would suggest that a mechanism other than Ca^{2+} entry might be involved in this process. Therefore, if we assume that, in response to different types of stimulation, release by exocytosis involves similar cellular and molecular mechanisms, the possibility of an increase in intracellular Ca^{2+} during Na$^+$-omission must be considered. Recently published results which show that iontophoretic administration of Ca^{2+} into nerve terminals (Miledi, 1973) and into mast cells (Kanno, Cochrane & Douglas, 1973) induces acetylcholine and histamine release respectively, seem to indicate that an increase in the intracellular levels of Ca^{2+} is a necessary event in stimulus-secretion coupling. Therefore, if the omission of Na^+ from the extracellular environment induces exocytotic release of catecholamines by increasing intracellular Ca^{2+}, the lack of a blocking effect of D-600 is to be expected.

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ON THE ACTIONS OF COMPOUNDS RELATED TO DOPAMINE AT A NEUROSECRETORY SYNAPSE

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1 The effects of a number of substances related to dopamine, including all its methylated derivatives, were investigated on the membrane potential and response to nerve stimulation of cockroach salivary gland cells.

2 Only *N*-methyldopamine (epinine), *N,N*-dimethyldopamine and *N,N*-dimethylnoradrenaline, all with unsubstituted hydroxyl groups, directly resembled dopamine in producing a hyperpolarization which could be as large as that caused by maximal nerve stimulation. During the continued presence of these substances the hyperpolarization waned and responses to nerve stimulation declined.

3 Many of the compounds caused one or both of two other effects, namely an increase in the rate of 'spontaneous miniature hyperpolarizations' and an enhancement of the submaximal responses to single nerve stimuli. There were no obvious structural requirements for these effects.

Introduction

Investigations of the pharmacology of dopamine-like substances have become of increasing interest in view of the likelihood that dopamine is a central neurotransmitter (see e.g. Hornykiewicz, 1966; Woodruff, 1971; Hornykiewicz, 1973; Vogt, 1973; Snyder, Banerjee, Yamamura & Greenberg, 1974; Iversen, 1975). However, the information obtained from the vertebrate central nervous system is in general somewhat indirect and it is evidently desirable to have available simpler systems in which physiological responses can be monitored directly. Several dopamine-sensitive preparations have recently been explored including the dog renal artery (see Goldberg, 1972) and certain ganglion cells in the mollusc (Woodruff & Walker, 1969; Ascher, 1972; Berry & Cottrell, 1975) and the guinea-pig (Hirst & Silinsky, 1975). A useful preparation may also be provided by the cockroach salivary gland (House, 1973) where there is growing evidence for dopaminergic transmission (Bland, House, Ginsborg & Laszlo, 1972; House, Ginsborg & Silinsky, 1973; Fry, House & Sharman, 1974; Ginsborg, House & Silinsky, 1974; Bowser-Riley & House, 1976) and this paper is concerned with the effects on that preparation of substances chemically related to dopamine.

The purpose of the work was first to obtain more information about the specificity of insect dopamine receptors, and secondly to explore the effects of a range of substances which might be formed from dopamine by metabolic processes. The substances investigated (which are listed in Table 1) include all the derivatives which result from methylation of the nitrogen and the oxygen atoms of dopamine. The experiments consisted in impaling acinar cells with a micro-electrode and observing the effect of the substance under test on the membrane potential and on the responses to nerve stimulation.

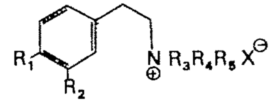
Methods

Compounds

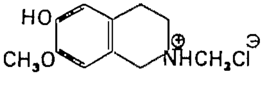
The amines tested (see Table 1) were generally crystallized as their hydrochloride salts from ethanol/ether and samples of 100–150 mg were analysed gravimetrically for halide. Melting points were recorded on a Mettler FP1 instrument at a heating rate of 0.2°C/minute.

Dopamine hydrochloride (1, Table 1) was obtained from Koch-Light and re-crystallized. 3-hydroxy-4-methoxyphenethylamine hydrochloride (2) was prepared from isovanillin by reaction with nitromethane to give the ω-nitro-styrene (Baxter, Allan & Swan, 1965) which was then reduced with lithium aluminium hydride (Fennoy, 1961). 4-Hydroxy-3-methoxyphenethylamine hydrochloride (3) was prepared by a similar method from vanillin. 3,4-Dimethoxyphenethylamine was obtained from Koch-Light and converted to its hydrochloride (4).

Table 1 Melting points and analyses of compounds



No.	*Trivial name*	*R_1*	*R_2*	*R_3*	*R_4*	*R_5*	*X^-*	*Melting-point (°C)*	*X^-* found	*X^-* calculated
1	Dopamine	OH	OH	H	H	H	Cl^-	242.7–243.5 (dec.)	18.61	18.69
2	4-Methyldopamine	OCH_3	OH	H	H	H	Cl^-	204.7–206.1	17.00	17.41
3	3-Methyldopamine	OH	OCH_3	H	H	H	Cl^-	212.1–213.0	17.27	17.41
4	3,4-Dimethyldopamine	OCH_3	OCH_3	H	H	H	Cl^-	153.5–154.2	16.25	16.29
5	Epinine	OH	OH	CH_3	H	H	Br^-	166.4–167.2	31.98	32.21
6	4-Methylepinine	OCH_3	OH	CH_3	H	H	Cl^-	156.3–158.2	16.51	16.29
7	3-Methylepinine	OH	OCH_3	CH_3	H	H	Cl^-	162.6–163.7	16.18	16.29
8	3,4-Dimethylepinine	OCH_3	OCH_3	CH_3	H	H	Cl^-	138.2–138.7	15.20	15.30
9	*N,N*-Dimethyldopamine	OH	OH	CH_3	CH_3	H	Cl^-	123.2–125.0	16.16	16.29
10	4-Methyl-*N,N*-dimethyldopamine	OCH_3	OH	CH_3	CH_3	H	Cl^-	161.1–162.7	15.24	15.30
11	3-Methyl-*N,N*-dimethyldopamine	OH	OCH_3	CH_3	CH_3	H	Cl^-	189.0–190.5	15.23	15.30
12	3,4-Dimethyl-*N,N*-dimethyldopamine	OCH_3	OCH_3	CH_3	CH_3	H	Cl^-	192.0–193.6	14.32	14.43
13	Coryneine (*N,N,N*-trimethyldopamine)	OH	OH	CH_3	CH_3	CH_3	I^-	204.7–205.0 (dec.)	39.22	39.27
14	4-Methyl-*N,N,N*-trimethyldopamine	OCH_3	OH	CH_3	CH_3	CH_3	I^-	215.5–215.9 (dec.)	37.35	37.63
15	3-Methyl-*N,N,N*-trimethyldopamine	OH	OCH_3	CH_3	CH_3	CH_3	I^-	*ca.* 187–190.8	37.57	37.63
16	3,4-Dimethyl-*N,N,N*-trimethyldopamine	OCH_3	OCH_3	CH_3	CH_3	CH_3	I^-	229.4–230.2	36.11	36.13
17	Methyl-*m*-tyramine	H	OCH_3	H	H	H	Br^-	126.4–127.5	34.45	34.42
18	*N,N*-Dimethyl-*m*-tyramine	H	OH	CH_3	CH_3	H	Cl^-	159.5–162.0	17.34	17.58
19	Methyl-*N,N*-dimethyl-*m*-tyramine	H	OCH_3	CH_3	CH_3	H	Cl^-	133.1–134.1	16.34	16.44
20	Hordenine	OH	H	CH_3	CH_3	H	Cl^-	169.0–170.7	16.75	17.58
21	Methyl-hordenine	OCH_3	H	CH_3	CH_3	H	Cl^-	173.5–175.6	16.41	16.44
22	Hordenine methiodide	OH	H	CH_3	CH_3	CH_3	I^-	226.5–227.1	41.28	41.31



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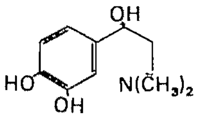
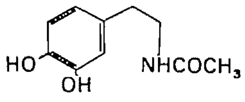


N,N-dimethylnoradrenaline
24



N-acetyldopamine
25

Difficulty was encountered in reducing the *N*-methyl amide of homoveratric acid with lithium aluminium hydride to give epinine (3,4-dihydroxyphenethylmethylamine, *N*-methyl dopamine); the ethyl ester was therefore reduced to the corresponding alcohol, which was converted to the chloride with phosphorus trichloride. Treatment with methylamine and demethylation with hydrobromic acid then gave epinine hydrobromide (5). For 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-phenethylmethylamine hydrochlorides (6 and 7) isovanillin and vanillin, respectively, were first protected by benzylation (Baxter *et al.*, 1965) converted to their ω-nitrostyrenes and reduced with lithium aluminium hydride to the primary amines. These were then converted to the *N*-monomethyl compounds by reaction with benzaldehyde, *N*-methylation with dimethyl sulphate and hydrolysis of the benzal group (Kirkwood & Marion, 1950). Finally, the benzyl group was removed by hydrogenolysis.

3,4-Dihydroxyphenethyldimethylamine hydrochloride (9) was obtained from homoveratric acid. The acid was converted to its chloride, which was reacted with dimethylamine and the resulting dimethylamide was reduced with lithium aluminium hydride. For 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxy-phenethyl-dimethylamine hydrochlorides (10 and 11), the protected primary amines prepared from isovanillin and vanillin (see above) were methylated with formaldehyde and formic acid (Moore, 1962). The products, however, included some tetrahydroisoquinoline derivatives formed by Pictet-Spengler ring closure, which were difficult to separate completely from the required products. This difficulty was avoided by carrying out the methylation reaction on 3-benzyloxy-4-methoxyphenethylmethylamine and its isomer, prepared above.

The quaternary derivative of dopamine, 3,4-dihydroxyphenethyl-trimethyl ammonium (13), was prepared by reaction of homoveratrylamine with methyl iodide followed by demethylation with hydriodic acid. The corresponding 4-methoxy and 3-methoxy compounds (14 and 15) were prepared by reaction of the tertiary base, in dry ether, with methyl iodide.

The tetrahydroisoquinoline obtained by Pictet-Spengler cyclisation of 3-benzyloxy-4-methoxyphenethylamine, presumably (after hydrogenolysis) 6-hydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (23), had m.p. 260.8–261.4°C, and contained 15.43% Cl^- (calculated content = 15.43%).

1-(3,4-Dihydroxyphenyl)-2-dimethylaminoethanol (*N,N*-dimethyl-noradrenaline, (24)) was prepared from catechol. Chloroacetylation (Fellman, 1957) followed by reaction with dimethylamine and reduction with sodium borohydride (Chapman, Clarke & Harvey, 1971) gave the required product which was crystallized as the free amine, m.p. 138.2–138.4°C (dec) (lit. value 142–3°C, La Manna & Campiglio, 1960).

N-acetyldopamine (25) was prepared from protocatechualdehyde, which was protected by benzylation and converted to the ω-nitro-styrene (Baxter *et al.*, 1965). Reduction with lithium aluminium hydride gave 3,4-dibenzyloxyphenethylamine which was isolated as its hydrochloride. Acetylation of the free amine followed by removal of the benzyl groups by hydrogenolysis gave an oil which could not be crystallized but which was homogenous on silica gel thin-layer chromatography.

Preparation

Salivary glands, together with their ducts, (in which the nerves are embedded) and reservoirs were dissected from the cockroach *Nauphoeta cinerea* Olivier. The glands were spread across a pedestal in a perspex bathing chamber of 4 ml capacity. The ducts were drawn into a suction electrode for stimulation of the encapsulated nerves. Microelectrodes were filled with 3 M KCl and had resistances from 10–30 MΩ. The output of a preamplifier (W.P. Instruments) was fed in parallel to a Gould-Brush 220 pen recorder and a Tektronix Model 565 cathode-ray oscilloscope. The records shown are photographs of pen recorder traces.

The normal bathing solution contained (mM) NaCl 160, $CaCl_2$ 5, KCl 1, $NaHCO_3$, 1 and NaH_2PO_4, 1 and was supplied to the preparation continuously at a rate of 2 ml/min by a Watson Marlow flow inducer and removed at the same rate by suction. Drugs were applied by changing the delivery tube of the flow inducer from the control to the drug solution and briefly increasing the flow rate to about 20 ml/minute.

Responses

The main features have already been described (House, 1973) but it may be convenient for the reader to have a brief account here. The response to nerve stimulation occurs after a delay of about 1 s and consists of a hyperpolarization whose amplitude and duration are graded with the strength and number of stimuli, sometimes followed by a small depolarization. In this investigation we have been concerned only with the hyperpolarization. The stimuli were of 0.5 ms duration and at a frequency of 90 hertz. Responses of maximal amplitude required up to 20 stimuli at 40 volts. The responses were approximately constant if the bursts of stimuli were repeated not more frequently than 1 per 2 minutes.

Results

Direct action on membrane potential

Each of the 25 compounds listed in Table 1 was tested on at least 2 preparations in concentrations of up to

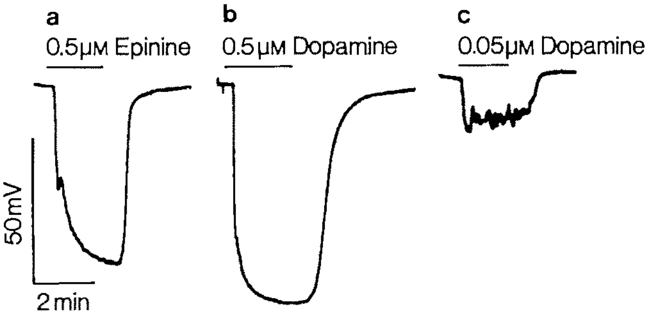


Figure 1 Hyperpolarizations recorded intracellularly from a cockroach salivary gland in response to epinine (0.5 µM) in (a) and dopamine (0.5 µM) in (b) and (0.05 µM) in (c) (same impalement). The periods in which the drug containing solutions flowed through the bath, replacing the flowing control solution (see methods section), are indicated by the lines above each trace.

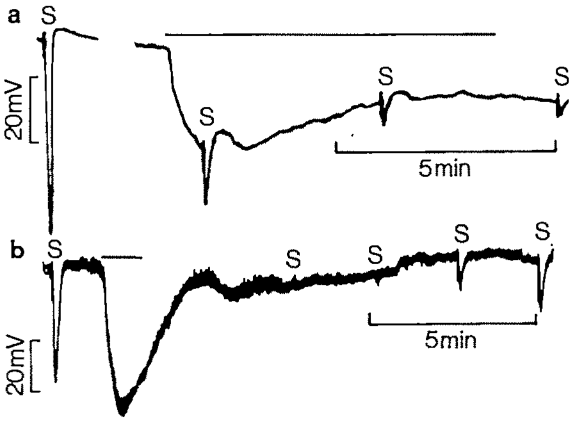


Figure 2 Waning of hyperpolarization and reduction of responses to nerve stimulation. Records from two different experiments in which *N,N*-dimethyl dopamine was admitted to the chamber in concentration of 0.4 µM (a) and 2 µM (b). The periods during which the drug solution was present are indicated by the lines above the traces. Bursts of nerve stimuli indicated by S: in (a) each burst consisted of 10 stimuli at 50 V (0.5 ms, 90 Hz) and in (b), of 4 stimuli at 20 V (0.5 ms, 90 Hz).

and including 10 µM, which is of the order of 100 times the concentration of dopamine needed for a half maximal response. Only 4 compounds brought about a hyperpolarization similar to that produced by a burst of nerve stimuli. These were dopamine (see also House *et al.*, 1973), epinine, *N,N*-dimethyldopamine, and, like noradrenaline (see Bland *et al.*, 1972), *N,N*-dimethylnoradrenaline. Thus from a structure activity point of view it appears that dopamine-like activity on the cockroach salivary gland survives mono- or dimethylation of the nitrogen atom but requires both unsubstituted catechol hydroxyl groups. Their presence is evidently however insufficient to produce dopamine-like activity, since *N*-acetyl-dopamine (a metabolite thought to be of greater importance in insects than in mammals, see Fänge & Hanson, 1973) is inactive.

Figure 1 illustrates the action of epinine and shows that its potency is somewhat less than that of dopamine (perhaps about one third). The potency of *N,N*-dimethyldopamine was found to be about the same as that of epinine, but more exhaustive experiments would be required to obtain reliable values.

Reduction of response to nerve stimulation

Figure 2 illustrates another effect of substances which cause hyperpolarization. During prolonged exposure to *N,N*-dimethyl-dopamine (Figure 2a), or even during short exposure to high concentrations (Figure 2b), the hyperpolarization gradually waned and the responses to nerve stimulation were reduced in amplitude

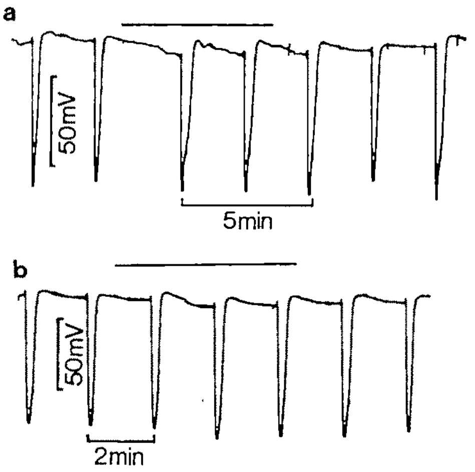


Figure 3 Absence of inhibitory effect on responses to maximal nerve stimulation. In (a) the responses are to bursts of 10 stimuli (0.5 ms, 90 Hz, 40 V); solution containing 20 µM coryneine admitted during period indicated by line above trace. In (b) responses were to 5 stimuli (0.5 ms, 90 Hz and 20 V) and the drug solution contained 20 µM 3-methyl-*N,N*-dimethyldopamine.

(Figure 2a) or sometimes even temporarily abolished (Figure 2b). Similar results have been obtained with all the hyperpolarizing substances so far tested on this preparation.

Absence of antagonist action

Apart from the action of the agonists described in the previous section, no other compounds had any blocking effect on the responses to nerve stimulation. Two experiments to test this point are illustrated in Figure 3. Bursts of stimuli were applied to the salivary nerves at regular intervals, the stimulus strength being chosen so that the responses were of maximal amplitude. In the experiment of Figure 3a the preparation was exposed to 20 µM coryneine (13, Table 1) and in 3b, to 20 µM 3-methyl-*N,N*-dimethyldopamine (11, Table 1): in neither case was there any reduction in response. Similar results were obtained with all the compounds of Table 1, except those causing hyperpolarization.

Enhancement of spontaneous activity

It has been reported by House (1973) that spontaneous electrical activity may occasionally be recorded from unstimulated salivary gland cells. This has the appearance of a random sequence of small hyperpolarizations (which have been referred to as miniature secretory potentials) varying in amplitude but similar in time course to the larger hyperpolarizations which result from nerve stimulation. In previous investigations, a number of substances (e.g. bretylium, Silinsky, 1974; tyramine, Ginsborg, House and Silinsky, unpublished observations) have been found to enhance this activity, and it has been tentatively suggested that these substances do so by promoting the release of packets of the neurotransmitter from the salivary nerve terminals. Several of the compounds of the present series enhanced spontaneous activity. The effect appears to be somewhat variable, perhaps because it depends on the particular state of the nerve terminals which may vary in a way not controlled experimentally. Figure 4 illustrates the effect of 10 µM *N,N*-dimethyl-*m*-tyramine from an experiment on a particularly responsive preparation. Other substances which enhanced spontaneous activity were 3-methylepinine, 3-methyl-*N,N,N*-trimethyldopamine and hordenine (7, 15 and 20, Table 1). It seems possible that the hyperpolarizing substances also produce this kind of activity superimposed on their direct effect; this would account for the increased 'noise' recorded during the responses to their application, especially where, as in Figure 1c for example, the responses are small, and the membrane potential distant from the equilibrium potential for the action of the agonist (see Ginsborg *et al.*, 1974).

Potentiation of submaximal responses

As was mentioned in the Methods section, it is generally necessary to apply a short burst of stimuli to the salivary nerve to produce a maximal hyperpolarization of the gland cells. With single stimuli submaximal responses occur: these have been found to be enhanced by many of the substances of the present series. Figure 5 illustrates the action of the quaternary derivative, coryneine (13, Table 1). Shortly after the control bathing solution was changed to one containing the drug, in a concentration of 10 µM, the responses to single stimuli increased in amplitude. In the experiment of Figure 5a, the enhanced response corresponded to that produced by a burst of between 3 and 4 stimuli in the control solution, and in 5b to that produced by 2 stimuli. It must be noted that the effect, like that of the enhancement of spontaneous activity described above, was variable. For example, in one experiment 10 µM coryneine had no effect although in three other similar experiments an unequivocal potentiation occurred.

The following compounds were observed to cause a potentiation at a concentration of 10 µM such that a single stimulus caused a response at least equivalent to that produced by two stimuli in the control solution: *dopamine derivatives:-* 3-methyl, 4-methyl; *epinine derivatives:-* 4-methyl, 3,4-dimethyl; N,N-*dimethyldopamine derivatives:-* 3-methyl, 3,4-dimethyl;

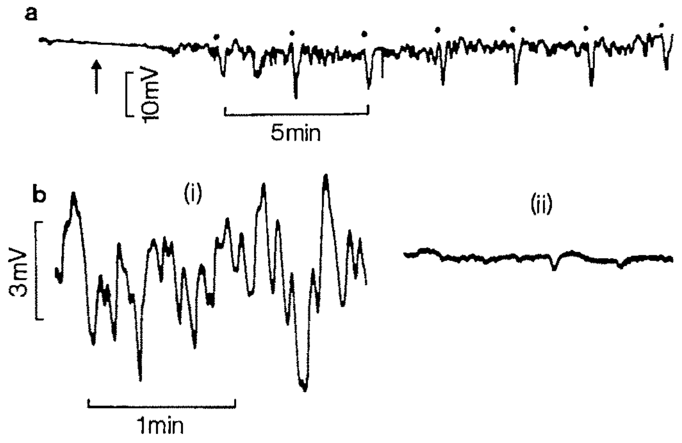


Figure 4 Enhanced spontaneous activity caused by 10 μM *N,N*-dimethyl-*m*-tyramine. In (a) the drug solution was admitted at the arrow. The symbol (●) above the trace indicates response to single stimuli (0.5 ms, 60 V). In (b) (i) from a different cell in the same preparation, the effect of 10 μM *N,N*-dimethyl-*m*-tyramine at higher amplification is shown; (ii) was recorded 10 min after the control solution was reinstated.

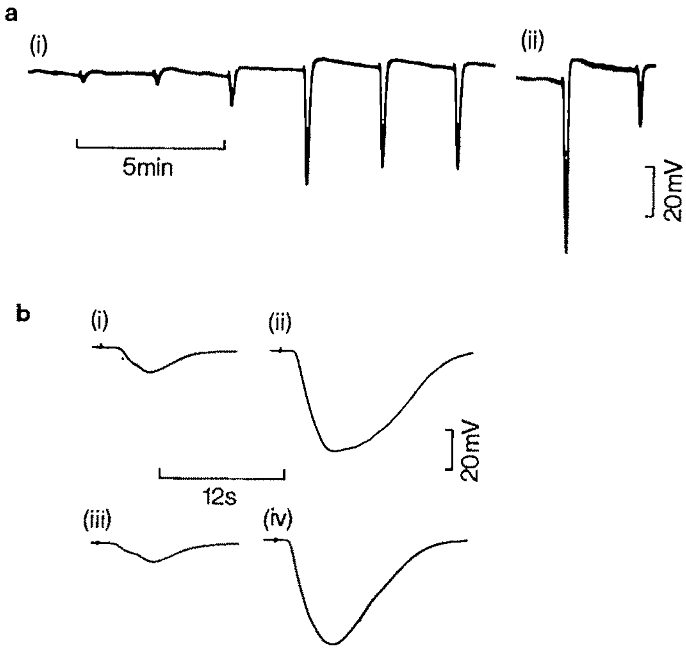


Figure 5 Effect of 10 μM coryneine on the responses to single stimuli. Two different preparations. In (a) (i) the responses are to 6 successive single stimuli of 15 volts. The drug solution was admitted immediately before the second stimulus. In (ii) responses after a prolonged wash in control solution to bursts of 4 and 3 stimuli respectively are shown. In (b), control responses to single stimuli of 10 V are shown in (i) and (iii); (ii) is the response to a single stimulus in 10 μM coryneine and (iv) the response to 2 stimuli in the control solution.

N,N,N-*trimethyldopamine and derivatives:-* coryneine, 3-methyl, 4-methyl, 3,4-dimethyl; *hordenine methiodide.* There are no obvious structural requirements: furthermore, because of the small number of experiments made with each compound (usually 2 or 3) it cannot be confidently assumed that the unlisted ones are necessarily always without effect.

Discussion

Three kinds of effect have been described in the preceding results. First are the hyperpolarizations which can be as large as those produced by maximal nerve stimulation. They are presumably due to a direct action on 'dopamine receptors' leading to an increase in potassium conductance (Ginsborg *et al.*, 1974). Such effects were produced only by those compounds with unsubstituted hydroxyl groups. This seems to be a general requirement for the retention of dopamine-like activity in simple dopamine analogues in a variety of situations (Goldberg, Sonneville & McNay, 1968; Woodruff & Walker, 1969; Sheppard & Burghardt, 1974; Miller, Horn, Iversen & Pinder, 1974; Costall, Naylor & Pinder, 1974) summarized in Table 2.

The second and third effects are an increase in the frequency of the small intermittent hyperpolarizations which occur spontaneously and the enhancement of responses to nerve stimulation. It may tentatively be suggested that they represent respectively enhancement of the spontaneous release of the neurotransmitter and an enhancement of its release by nerve stimulation (cf. Von Voigtlander & Moore, 1973). It must however be stressed that there is no direct evidence relating to these suggestions and that experiments of a different kind will be required to test them. A post-synaptic effect cannot at present be ruled out. On the assumption that the present results are relevant to the central nervous system, it is of interest to consider their possible bearing on the theories which attribute certain psychoses to abnormal metabolites (see e.g. Ernst, 1965) which over-stimulate dopaminergic systems (see e.g. Snyder *et al.*, 1974; Matthysse & Lipinski, 1975). Of the three possible forms of stimulation which might be produced by methylated dopamine metabolites, it seems unlikely that the direct effect of the *N*-methylated derivatives would be of importance, since a persistent concentration of either would inhibit the effect of dopaminergic nerve stimulation (see Figure 2). Furthermore they would presumably be subject to the normal enzymatic de-activation process of 3-methylation. However many of the 3-methylated derivatives produce the two effects that appear to enhance dopaminergic activity and might therefore be harmful.

We are greatly indebted to Dr R.B. Barlow for much help and advice. K.W.T. was a Mental Health Trust and Research Fund Fellow. Animal accommodation was provided by the Wellcome Research Unit.

Table 2 Comparison of effects of various dopamine analogues in different tests

	Test situation and reference	*Substances tested, also tested in this paper*	*Effect*	*Effect this paper*
			D = dopamine-like	
1	Renal vasodilatation (Goldberg *et al.*, 1968)	Epinine	D	D
		N,N-dimethyldopamine	–	D
		3-Methyldopamine	–	–
		4-Methyldopamine	–	–
2	Inhibition in snail brain (Woodruff & Walker, 1969)	Epinine	D	D
		3-Methyldopamine	–	–
		3,4-Dimethyldopamine	–	–
3	Activation of dopamine-sensitive adenylate cyclase from rat brain (Miller *et al.*, 1974)	Epinine	D	D
		N,N-dimethyldopamine	D	D
		Coryneine	D	–
		3-Methyldopamine	–	–
		4-Methyldopamine	–	–
	(Sheppard & Burghardt, 1974)	Epinine	D	D
		N,N-dimethyldopamine	D	D
		Coryneine	–	–
4	Behaviour changes in the rat (Costall *et al.*, 1974)	Epinine	D	D
		N,N-dimethyldopamine	D	D
		3-methyldopamine	–	–
		4-methyldopamine	–	–

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EVIDENCE CONCERNING THE INVOLVEMENT OF 5-HYDROXYTRYPTAMINE IN THE LOCOMOTOR ACTIVITY PRODUCED BY AMPHETAMINE OR TRANYLCYPROMINE PLUS L-DOPA

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1 Pretreatment of rats with *p*-chlorophenylalanine (PCPA; 2 × 200 mg/kg) decreased the concentration of 5-hydroxytryptamine (5-HT) in the brain. It also decreased the locomotor activity produced by tranylcypromine plus L-DOPA administration 24 h after the second dose of PCPA.

2 Pretreatment with *p*-chloroamphetamine, which produced a similar decrease in brain 5-HT concentrations did not decrease the locomotor response to tranylcypromine and L-DOPA.

3 PCPA pretreatment decreased the rise in the concentration of DOPA and dopamine in the brain following tranylcypromine and L-DOPA, suggesting its effect on the dopamine-induced locomotor activity was the result of this drug diminishing dopamine formation in the brain, probably by inhibiting L-DOPA uptake.

4 The locomotor activity produced by tranylcypromine and L-DOPA was not decreased by pretreatment 6 h earlier with disulfiram (400 mg/kg). This argues against the locomotor activity being due to noradrenergic stimulation.

5 PCPA pretreatment did not alter amphetamine-induced stereotypy or the circling behaviour in unilateral nigro-striatal lesioned rats.

Introduction

When rats or mice have been pretreated with a monoamine oxidase (MAO) inhibitor, such as pargyline or tranylcypromine and are subsequently given a loading dose of L-DOPA they display various behavioural changes including enhanced locomotor activity. These effects have been termed the 'dopa potentiation test' (Everett, 1966). The test has been suggested as a useful model for the study of the action of some antidepressants (Everett, 1966).

Enhanced locomotor activity following monoamine oxidase inhibition plus L-DOPA administration is seen if the animals have been pretreated with either of the peptides thyrotropin releasing hormone (TRH) or melanocyte stimulating hormone release inhibitory factor (MIF) (Plotnikoff, Kastin, Anderson & Schally, 1971; Plotnikoff, Prange, Breese, Anderson & Wilson, 1972; Plotnikoff, Prange, Breese & Wilson, 1974; Green & Grahame-Smith, 1974a; Huidobro-Toro, Scotti de Carolis & Longo, 1974).

While it is likely that the behavioural changes seen after tranylcypromine and L-DOPA are partly due to stimulation of dopamine receptors in the brain, the fact that similar behavioural changes are also seen following tranylcypromine and L-tryptophan administration (Grahame-Smith, 1971) led Jacobs (1974) to suggest that some of these behavioural responses are due to increased functional activity of brain 5-hydroxytryptamine (5-HT), possibly as the result of DOPA being decarboxylated in 5-HT neurones. In support of this contention he reported that the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA) partly inhibited the tranylcypromine/L-DOPA-induced behavioural changes.

To be able to interpret the findings on the action of peptides on the 'DOPA potentiation test' it is therefore necessary to know whether the behavioural changes are due solely to dopamine stimulation or whether 5-HT is also involved.

Methods

Adult male Sprague-Dawley rats weighing 150–200 g (Anglia Laboratory Animals, Alconbury, Huntingdon) were used in all experiments, except those in which animals were lesioned (see below) when rats weighing 300 g were used. All drugs except disulfiram were given by intraperitoneal injection dissolved in 0.9% w/v NaCl solution (saline) or suspended in saline containing 1.0% carboxymethyl cellulose. Control rats were given the vehicle only. Disulfiram was dissolved in the minimum volume of acetone and the control rats received the acetone only. Drugs were obtained from the following sources: tranylcypromine (Smith, Kline & French), L-DOPA, 6-hydroxydopamine, *p*-chlorophenylalanine, *p*-chloroamphetamine, methamphetamine and disulfiram (Sigma Chemical Co.), apomorphine (MacFarlan-Smith).

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6-Hydroxydopamine lesions were made in rats anaesthetized with Equithesin (Jensen-Salsbery Labs). They were positioned in a stereotaxic apparatus and injected unilaterally with 6-hydroxydopamine hydrobromide (freshly dissolved in cold saline containing 1 mg/ml ascorbic acid) through a 30-gauge stainless steel needle aimed at the substantia nigra. The co-ordinates of the cannula tips were 2.8, 2.0, 8.0 according to the atlas of Pellegrino & Cushman (1967). Four µl of the 6-hydroxydopamine solution (2 mg base/ml) was injected at a rate of 1 µl/minute.

Brain 5-HT was measured by the method of Curzon & Green (1970) and noradrenaline and dopamine by the method of Chang (1964). L-DOPA was measured by the column chromatographic, fluorimetric technique described by Andén, Engel & Rubenson (1972).

Circling activity was measured in rotometer bowls of white translucent plastic supported on metal rings. Bowls measured 30 cm in diameter and 26 cm in height. Locomotor activity was measured on groups of three animals using LKB Animex activity meters (sensitivity and tuning: 30 µA) as described previously (Grahame-Smith, 1971; Green & Grahame-Smith, 1974b).

Results

Effect of p-*chlorophenylalanine on the locomotor activity following tranylcypromine and L-DOPA*

Rats were injected with *p*-chlorophenylalanine (PCPA; 200 mg/kg) or the vehicle on Day 1 and Day 2. Twenty-four hours after the second injection of PCPA both groups were given tranylcypromine (20 mg/kg); L-DOPA (100 mg/kg) was administered after a further 30 minutes.

Following tranylcypromine and L-DOPA the rats displayed irritability, reactivity, squeaking, aggression, forepaw padding, headweaving, tremor, salivation, piloerection, locomotor activity and rearing to other animals.

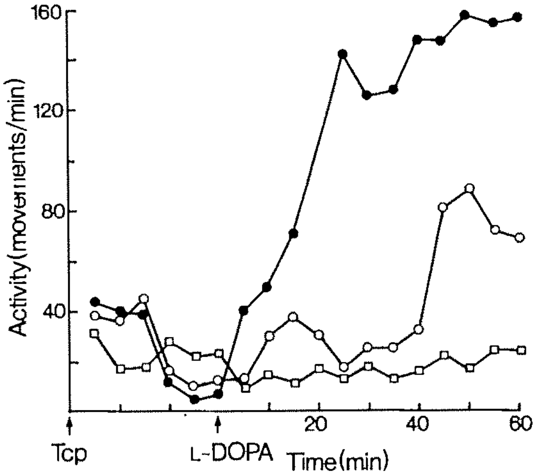

Figure 1 Effect of *p*-chlorophenylalanine (PCPA) on the locomotor activity produced by tranylcypromine and L-DOPA administration. Rats were injected with PCPA (200 mg/kg) or vehicle on Day 1 and Day 2. On Day 3, 24 h after the second injection both groups were injected with tranylcypromine (Tcp; 20 mg/kg) and after a further 30 min with L-DOPA (100 mg/kg). Activity of vehicle-injected group (●), PCPA-treated (○) group and control group given tranylcypromine only (□).

The animals that had been pretreated with PCPA no longer showed rigidity, forepaw padding or tremor and headweaving and salivation were much decreased. Aggression and rearing to other animals was still sometimes seen. The locomotor activity now more closely resembled that seen after low doses of methamphetamine (1–2 mg/kg) and was much reduced in comparison to control animals (Figure 1).

Biochemical measurements showed that at the start of the experiment the brain 5-HT concentration in the PCPA-treated rats was decreased by 75% (Table 1). Noradrenaline concentrations were unaltered but there was a small decrease in dopamine following PCPA.

In rats given tranylcypromine and L-DOPA it was found that 90 min after L-DOPA administration, the PCPA-treated rats had a lower brain dopamine concentration than controls. This was presumably because the L-DOPA concentration in the brain following tranylcypromine and L-DOPA is decreased by pretreatment with PCPA (Table 2).

Effect of p-*chloroamphetamine on the locomotor activity following tranylcypromine and L-DOPA*

p-Chloroamphetamine (PCA) inhibits 5-HT synthesis leading to prolonged brain 5-HT depletion (Sanders-Bush, Gallager & Sulser, 1974) although it also has

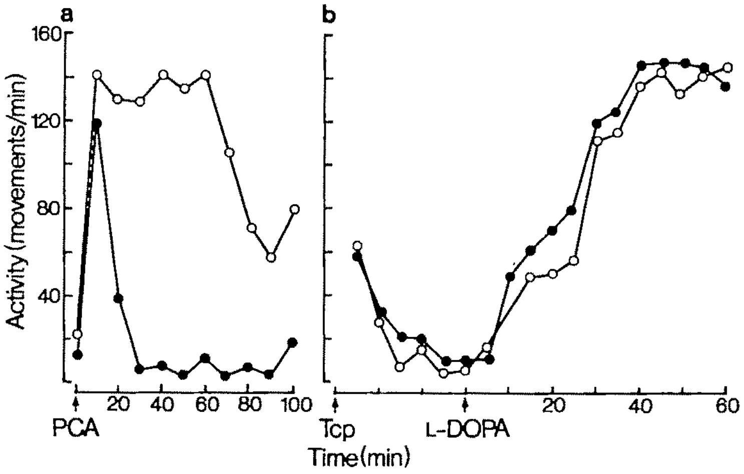

Figure 2 Effect of *p*-chloroamphetamine (PCA) on locomotor activity and activity following tranylcypromine and L-DOPA administration. Rats were injected with saline (●) or PCA, 10 mg/kg (○). (a) Shows effect of these treatments on locomotor activity immediately after injection. (b) Shows locomotor activity when both groups were injected with tranylcypromine (Tcp; 20 mg/kg) followed by L-DOPA (100 mg/kg), 24 h after the PCA injection.

several other actions on brain amine metabolism (see Fuller & Molloy, 1974). It was used in the present study to decrease brain 5-HT and investigate whether this resulted in decreased locomotor activity following tranylcypromine and L-DOPA administration.

Rats were injected with saline or PCA (10 mg/kg). In agreement with previous reports (Frey & Magnussen, 1968; Buus Lassen, 1974) the PCA-treated animals rapidly became hyperactive and behaviourally were identical to rats given tranylcypromine and L-tryptophan (Figure 2a). This effect lasted for over 2 h and will be discussed later. Twenty-four hours after the PCA injection brain 5-HT was greatly decreased, while noradrenaline and dopamine concentrations were unaltered (Table 1). The PCA and saline-injected animals were then given tranylcypromine (20 mg/kg) and after a further 30 min L-DOPA (100 mg/kg). PCA-pretreatment had no effect on the locomotor activity (Figure 2b). Behaviourally they were also very similar to the control animals except that they seemed to be more reactive both to the other rats in the cage, jumping violently when they touched each other, and to outside stimuli such as noise. These features appeared much later after L-DOPA in the control rats. PCA-pretreatment did not alter the increase in dopamine and noradrenaline concentrations, seen after tranylcypromine and L-DOPA administration (Table 2).

Table 1 Effect of *p*-chlorophenylalanine (PCPA) and *p*-chloroamphetamine (PCA) on rat brain 5-hydroxytryptamine (5-HT), dopamine and noradrenaline

	Brain amine concentrations (μg amine/g brain (wet wt.))		
Injected	*5-HT*	*Dopamine*	*Noradrenaline*
Saline	0.51 ± 0.01 (4)	1.46 ± 0.08 (8)	0.24 ± 0.03 (6)
p-Chlorophenylalanine (200 mg/kg × 2)	0.14 ± 0.02 (4)*	1.14 ± 0.11 (4)	0.27 ± 0.02 (6)
p-Chloroamphetamine (10 mg/kg)	0.18 ± 0.02 (6)*	1.27 ± 0.04 (4)	0.28 ± 0.02 (4)

Rats were injected with PCPA 200 mg/kg on Day 1 and 2; 24 h later they were killed for brain amine determinations. PCA (10 mg/kg) was injected 24 h before rats were killed and brain amines determined. Results show mean ± s.e. mean with number of determinations in brackets. *Different from control ($P < 0.001$).

Effect of p-*chlorophenylalanine on activity following methamphetamine*

These results suggested that PCPA altered the tranylcypromine/L-DOPA activity by inhibiting L-DOPA uptake into the brain and not because of alterations in brain 5-HT concentrations. We therefore next examined the effect of PCPA on locomotor activity or circling behaviour. These effects appear to depend on amphetamine-induced release of dopamine (Ungerstedt, 1971; Thornburg & Moore, 1973; Kelly, Seviour & Iversen, 1975; Kelly, 1975) and are independent of L-DOPA uptake.

Rats were injected with vehicle or PCPA (200 mg/kg) on Day 1 and Day 2. Twenty-four hours after the second injection both groups were given methamphetamine (2 mg/kg or 5 mg/kg). The lower dose of methamphetamine produces locomotor stimulation whereas the higher dose results in stereotypy.

PCPA-pretreatment did not alter the locomotor activity produced by 2 mg/kg methamphetamine (total movements over 90 min: controls; 9440 ± 150 (3), experimental; 9500 ± 220 (3)) nor did PCPA alter the degree of stereotypy observed when methamphetamine (5 mg/kg) was given.

The effect of PCPA-pretreatment on the methamphetamine-induced rotational behaviour of unilaterally nigro-striatal lesioned rats was also observed. The lesioned rats were tested at least twice with methamphetamine (5 mg/kg) in control experiments and the number of contralateral turns did not alter significantly. They were then injected with PCPA (200 mg/kg) on two successive days. On the third day, 24 h after the final injection they were again given methamphetamine (5 mg/kg) and circling

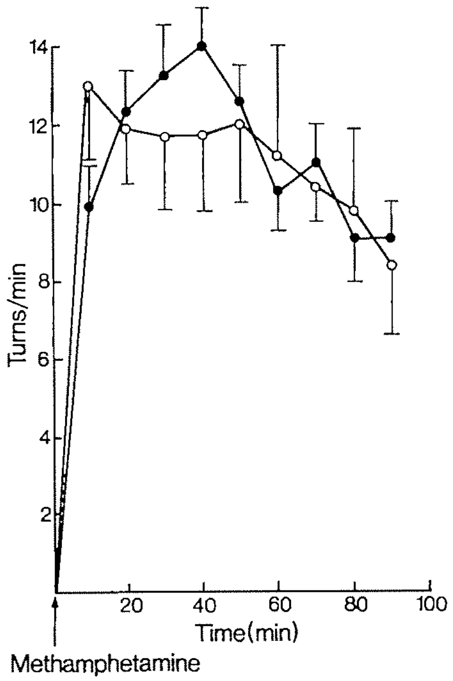

Figure 3 Effect of *p*-chlorophenylalanine (PCPA) on circling behaviour of unilateral nigro-striatal lesioned rats. Lesioned rats (see methods section) were tested at least twice with methamphetamine (5 mg/kg) in control experiments and the number of contralateral turns (which did not alter significantly from test to test) measured (●). They were then injected with PCPA (200 mg/kg) on two successive days. On Day 3 (24 h after the last PCPA injection) they were given methamphetamine (5 mg/kg) and number of turns measured (○). Vertical lines show s.e. mean.

Table 2 Effect of *p*-chlorophenylalanine (PCPA) and *p*-chloroamphetamine (PCA) on rat brain L-DOPA, dopamine and noradrenaline concentrations following subsequent tranylcypromine (Tcp) and L-DOPA administration

	Brain catechol concentrations (µg catechol/g brain (wet wt.))		
Injected	*L-DOPA*	*Dopamine*	*Noradrenaline*
Saline	0.78 ± 0.29 (4)	1.46 ± 0.08 (8)	0.24 ± 0.03 (6)
Tranylcypromine (20 mg/kg) + L-DOPA (100 mg/kg)	55.1 ± 7.4 (4)	11.74 ± 1.29 (7)	0.57 ± 0.05 (7)
PCPA (200 mg/kg × 2) + Tcp (20 mg/kg) + L-DOPA (100 mg/kg)	11.5 ± 4.0 (4)*	4.71 ± 0.61 (8)*	0.49 ± 0.04 (6)
PCA (10 mg/kg) + Tcp (20 mg/kg) + L-DOPA (100 mg/kg)	N.D.	9.10 ± 0.88 (6)	0.53 ± 0.03 (6)

Rats were injected with PCPA or PCA as described in footnote to Table 1. Twenty-four hours after the PCA or second PCPA injection they were injected with tranylcypromine (20 mg/kg) and after a further 30 min with L-DOPA (100 mg/kg). Rats were killed for brain noradrenaline and dopamine determinations 90 min after L-DOPA and after 60 min for L-DOPA determinations. Results show mean ± s.e. mean with number of determinations in brackets. N.D.—not determined.

* Different from tranylcypromine/L-DOPA values $P < 0.001$.

behaviour measured. No difference in the degree of turning was observed following PCPA-pretreatment (Figure 3).

Effect of disulfiram on tranylcypromine/L-DOPA locomotor activity

Because administration of tranylcypromine and L-DOPA results in a significant rise not only of dopamine but also of noradrenaline (Table 2) it seemed possible that some of the activity observed was due to noradrenergic stimulation. This was investigated by inhibiting the conversion of dopamine to noradrenaline with the dopamine β-hydroxylase inhibitor, disulfiram. The dose used has been shown to produce a 70% reduction in brain noradrenaline concentrations (Green & Grahame-Smith, 1974b).

Rats were injected with vehicle or disulfiram (400 mg/kg). Six hours later both groups were given tranylcypromine (20 mg/kg) with L-DOPA (100 mg/kg) after a further 30 minutes. No difference was observed between the activity of the two groups.

Discussion

In agreement with Jacobs (1974) we found that PCPA altered the behaviour following monoamine oxidase inhibition and L-DOPA administration. However, the biochemical results suggest that this effect may not be due to involvement of 5-HT in the behavioural syndrome, but may be an effect of PCPA-pretreatment on L-DOPA uptake and subsequent dopamine formation. The PCPA-treated rats displayed about 40% less activity than the controls and PCPA decreased dopamine concentrations in the brain by a similar value. Furthermore, PCA administration produced a similar decrease in brain 5-HT concentration but did not alter the increased rate of dopamine formation following tranylcypromine/L-DOPA administration and did not inhibit the locomotor activity.

While in the current experiments the last PCPA injection was given 24 h before L-DOPA administration and in the experiments of Jacobs (1974) it was given 72 h before the DOPA, there is evidence that PCPA would still be present in significant amounts in both brain and plasma at this time (Gal, Roggeveen & Millard, 1970). The decreased L-DOPA concentration in the brain may be due to the PCPA competing with this amino acid for uptake. PCPA and tryptophan compete for uptake into synaptosomes (Grahame-Smith & Parfitt, 1970) and the tryptophan concentration in the brain following a load is decreased in PCPA-treated rats (Grahame-Smith, 1971). However, the finding that tryptophan uptake into synaptosomes was decreased if the animals had been injected with PCPA 24 h earlier (Parfitt, 1972) raises the possibility that PCPA in the brain might block subsequent entry of other amino acids.

When dopamine was released from dopaminergic neurones by methamphetamine injection, PCPA-pretreatment failed to alter either the stereotypy in normal rats or circling behaviour in nigro-striatal lesioned animals, in agreement with other reports (Marsden & Guldberg, 1973; Breese, Cooper & Mueller, 1974).

Unlike Breese *et al.* (1974) we were unable to show enhanced locomotor activity following amphetamine in rats pretreated with PCPA. It is possible that this is the result of the different dose of amphetamine used (we used 2 mg/kg while they used 3 mg/kg). However, it should be pointed out that they found potentiation of marginal significance at a dose of 1 mg/kg. Jacobs, Wise & Taylor (1975) concluded on the basis of their studies that while PCPA-pretreatment may result in amphetamine-treated rats displaying higher activity than control animals, the significance is doubtful as the PCPA-injected rats also have greater spontaneous locomotor activity (Fibiger & Campbell, 1971). We feel that our results together with those of Jacobs *et al.* (1975) seriously weaken the concept of an amphetamine-5-HT interaction or a 5-HT involvement in dopamine-induced locomotor activity produced by tranylcypromine/L-DOPA administration. Quock & Horita (1974) observed no alteration in behavioural responses of rabbits given the dopamine agonist apomorphine after depletion of 5-HT by PCPA. It should be noted, however, that raphé lesions have been reported to alter responses to agonists acting on dopamine receptors (Costall & Naylor, 1974).

While Dolphin, Jenner & Marsden (1975) have shown involvement of noradrenaline in L-DOPA reversal of reserpine-induced akinesia, a noradrenergic component of the tranylcypromine/L-DOPA hyperactivity was not demonstrated.

Decarboxylation of 5-hydroxytryptophan (5-HTP) and L-DOPA is performed by the same enzyme (Kuntzman, Shore, Bogdanski & Brodie, 1961). Loading doses of L-DOPA result in catecholamine fluorescence in 5-HT neurones and 5-hydroxytryptophan (5-HTP) loading produces 5-HT fluorescence in catecholamine neurones (Fuxe, Butcher & Engel, 1971; Butcher, Engel & Fuxe, 1972). It is often suggested, therefore, that administration of either of these precursors results in non-specific stimulation. However, the failure of PCA to decrease tranylcypromine/L-DOPA activity and α-methyl *m*-tyrosine to decrease 5-HTP-induced activity (Modigh, 1971) argues against this non-specific decarboxylation necessarily having functional significance.

Finally, we wish to suggest that the hyperactivity following PCA injection (Figure 2a) is not due to an amphetamine-like action of this drug in releasing dopamine as previously suggested (Strada, Sanders-Bush & Sulser, 1970; Strada & Sulser, 1971; Costa, Naimzada & Revuelta, 1971) but the result of 5-HT receptor stimulation. PCA releases 5-HT (Wong, Horng & Fuller, 1973), is a potent monoamine oxidase inhibitor (Fuller, 1966; Fuller & Hines, 1970) and inhibits 5-HT re-uptake (Carlsson, 1970; Wong *et at*., 1973). Therefore, when 5-HT is released it is not metabolized intraneuronally and 'spills-over' into the synaptic cleft producing stimulation of 5-HT receptors, explaining why the hyperactivity following PCA is identical to that seen after tranylcypromine/L-tryptophan administration; an observation also made by Jacobs, Mosko & Trulson (1976). It also explains why PCPA abolishes PCA-induced hyperactivity (Buus Lassen, 1974; Jacobs *et at*., 1976). α-Methyl *p*-tyrosine blocks PCA-induced hyperactivity (Buus Lassen, 1974) and 5-HT-induced hyperactivity is also inhibited by this drug (Green & Grahame-Smith, 1974b).

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THE EFFECT OF ASPIRIN ON 5-HYDROXYTRYPTAMINE UPTAKE AND RELEASE BY HUMAN PLATELETS

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The influence of aspirin on 5-hydroxytryptamine (5-HT) uptake and storage by human blood platelets has been investigated. Uptake of 5-HT was strongly inhibited. In 30 min aspirin released 50% of the 5-HT that had been incorporated into the platelets prior to the addition of the aspirin. These results are discussed in terms of possible interference with a 5-HT membrane receptor and the impairment of 5-HT storage in the dense granules.

Introduction Acetyl salicylic acid (aspirin) has been shown to be an effective inhibitor of certain platelet functions. Platelets exposed *in vivo* or *in vitro* to this drug show a normal primary aggregation when the inducer is adenosine 5′-diphosphate (ADP) or adrenaline. Moreover the secondary wave of aggregation is abolished. Aggregation induced by collagen is markedly reduced. It has thus been reported that aspirin inhibits the release reaction responsible for the secondary aggregation as induced by ADP or adrenaline (Zucker & Peterson, 1968; Weiss, Aledort & Kochwa, 1968) and causes cell membrane damage (O'Brien, 1969).

The work reported here describes the action of aspirin on 5-HT uptake by blood platelets. 5-HT is accumulated in platelets from the surrounding medium against a high concentration gradient. It is well established that the amine is taken up by the platelet by two different mechanisms: passive diffusion which reflects the concentration gradient of 5-HT outside and inside the platelet, and active uptake which requires energy and which leads to a saturation level of intracellular 5-HT (Born, Juengjaroen & Michal, 1972; Da Prada & Pletscher, 1972). Several investigations from different laboratories, most of which are studies on the smooth muscle, indicate that the 5-HT receptor may be a proteolipid (Woolley & Gommi, 1965; Marcus, Safier & Ullman, 1972; Heyningen, 1974).

The results obtained in this study clearly show that aspirin diminishes 5-HT uptake. Discussion is open as to whether the mechanism of aspirin inhibition of the amine uptake through the cell membrane could be responsible for its inhibitory effect on release.

Methods Blood was collected from normal human volunteers in a hypercitrated medium (1 volume for 10 volumes final) and centrifuged for 15 min at 100 *g*. The concentration of platelets in the platelet rich plasma (PRP) was determined by counting using a phase contrast microscope and was adjusted to 300 000 per mm^3 by dilution with autologous plasma free of platelets.

[^{14}C]-5-HT was obtained from C.E.A. (Saclay) and its uptake was measured as previously described by Rendu & Caen (1973). The final concentration of [^{14}C]-5-HT added to the PRP was 3.7 mCi/mM. To assess the inhibition of [^{14}C]-5-HT uptake by aspirin, PRP was preheated 5 min at 37°C and aspirin (Theraplix) was added at a concentration of 1.1 mM without shaking for 5 min before the incubation with [^{14}C]-5-HT. The uptake was stopped at increasing time intervals by rapid centrifugation in an Eppendorf microcentrifuge. The radioactivity in the platelet pellet was measured in an Intertechnique Model SL 4000 liquid scintillation counter by the external standard method with an efficiency of 80%. To assess the effect of aspirin on the storage of 5-HT, aspirin was also added 30 min after the addition of [^{14}C]-5-HT to the PRP with the incubation prolonged for a further 30 min period. All results are expressed as the percentage of the total [^{14}C]-5-HT added to the PRP which was incorporated within the platelet pellet.

The basic fluorescent dye mepacrine (K and K Labs) at a concentration of 50 μM was incubated with PRP for 30 min at 37°C. This dye allows the visualization of the storage bodies (Lorez, Da Prada & Pletscher, 1975). The platelets were sedimented, resuspended in saline and ACD A and examined by fluorescence microscopy using a photomicroscope (Zeiss) equipped with a super pressure lamp (HBO 200 W/4) with vertical illumination.

Results The rate of incorporation of [^{14}C]-5-HT by platelets in PRP is shown in Figure 1a. The concentration of [^{14}C]-5-HT added (5.4 μM) was such that the early part of the incorporation represented active uptake and the later part (after 30 min) passive diffusion (Da Prada & Pletscher, 1972). Pretreatment with aspirin resulted in a strong inhibition of the 5-HT incorporation. At a final concentration of 1.1 mM, aspirin inhibited both the rapid uptake and passive diffusion by 70%. The effect of aspirin on the storage of 5-HT that had already been incorporated in the platelets was also studied. PRP was incubated with

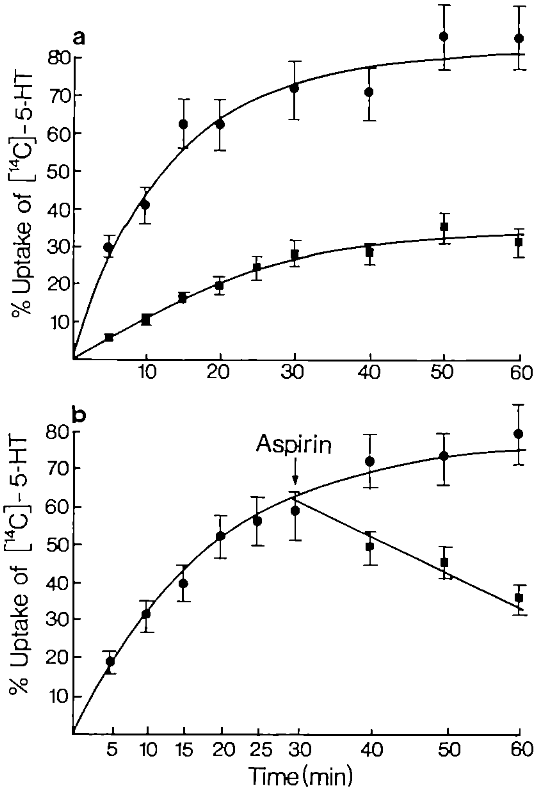

Figure 1 (a) The uptake of [^{14}C]-5-hydroxytryptamine (5-HT) by normal platelets (●) and by aspirin-(1.1 mM) treated platelets (■). (b) The uptake of [^{14}C]-5-HT by normal platelets (●) and the release of [^{14}C]-5-HT from platelets (■) after the addition of aspirin (1.1 mM).

[^{14}C]-5-HT for 30 minutes. Aspirin (1.1 mM) was added and the incubation prolonged for another 30 min period. Following the addition of the aspirin no further incorporation of [^{14}C]-5-HT was observed and there was a gradual decrease in the level of [^{14}C]-5-HT that had been incorporated; after 30 min incubation with aspirin 50% of the 5-HT that had been incorporated was liberated (Figure 1b).

Control platelets loaded *in vitro* with mepacrine (50 µM) and examined with the fluorescence microscope showed a green-yellow fluorescence which was concentrated in the dense granules. The drug thus enabled the visualization of the platelet 5-HT storage organelles. When the platelets were pretreated with aspirin before the addition of the mepacrine, the fluorescence observed in the dense granules was much weaker and was also diffused throughout the platelet.

Discussion Aspirin was observed to inhibit both the initial active and the later passive uptake of [^{14}C]-5-HT. This indicates that the action of the drug is complex, which is confirmed by the observations using mepacrine where in aspirin-treated platelets the fluorescence was diffused throughout the platelet cytoplasm and not only concentrated in or on the dense bodies. This suggests that aspirin not only has a surface effect and interferes with 5-HT uptake at the level of the receptor sites but also has a profound direct or indirect effect inside the platelet.

In addition, aspirin was unexpectedly observed to exert a 'releasing effect' which resulted in the liberation of at least 50% of the [^{14}C]-5-HT that had previously been incorporated. This aspirin-induced decrease in incorporated [^{14}C]-5-HT may be attributed to cell damage. It may, however, be a result of an impaired storage of the amine in the dense bodies, this also being compatible with the mepacrine fluorescence which is diffused throughout the platelet cytoplasm following aspirin pretreatment.

In many reports including those of Weiss *et al.* (1968); Zucker & Peterson (1968) and O'Brien (1969), the main action of aspirin on human platelets has been stated to be an inhibition of the release reaction. However, the results described here raise the question whether aspirin is really a release inhibitor. Al-Mondhiry, Marcus & Spaet (1970) have suggested that the action of aspirin may be at various sites in the cell. Recently it has been proposed that aspirin may impair the production of prostaglandins and their endoperoxide intermediates (Smith & Willis, 1971). Droller (1974) has also suggested that the inhibition of thrombin-induced cyclic adenosine 3′,5′-monophosphate formation in human platelets by aspirin is also related to an inhibition of prostaglandin synthesis. Thus the impairment of 5-HT uptake by platelets in the presence of aspirin may also be related to the inhibition of prostaglandin synthesis. Further investigations are needed to elucidate whether prostaglandin production has a role to play in 5-HT transport by blood platelets as has already been suggested by Sih, Takeguchi & Foss (1970).

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POTENTIATION BY DESIPRAMINE OF NEURONAL RESPONSES TO MESCALINE

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The effect of desipramine on responses of single cortical neurones to mescaline was studied by the microelectrophoretic technique. Both potentiation and antagonism of responses to mescaline by desipramine were observed. The antagonism may be related to the α-adrenolytic action of desipramine. The potentiation is unlikely to reflect the uptake blocking action of desipramine, since desipramine does not block the uptake of mescaline in the cerebral cortex. It is suggested that the potentiation may be due to a post-synaptic action of desipramine.

Introduction It is well known that the tricyclic antidepressant drugs (e.g. imipramine and desipramine) block the uptake of monoamines into nerve terminals (Horn, Coyle & Snyder, 1971). The drugs also potentiate pharmacological responses to the monoamines in the periphery and in the central nervous system (Sigg, Soffer & Gyermek, 1963; Bradshaw, Roberts & Szabadi, 1974). Although it is widely believed that there is a causal relationship between these two phenomena (Iversen, 1974), we have recently presented evidence that potentiation of responses to the monoamines can occur in situations where uptake blockade is unlikely to operate. For instance, desipramine can potentiate neuronal responses to noradrenaline and dopamine in the caudate nucleus (Bevan, Bradshaw & Szabadi, 1975a), although it does not block the uptake of catecholamines in this structure (Horn *et al.*, 1971). Furthermore, iprindole, a tricyclic antidepressant with little uptake-blocking activity (Ross, Renyi & Ögren, 1971) is effective in potentiating the responses of single cortical and caudate neurones to monoamines (Bevan, Bradshaw & Szabadi, 1975b). In this paper we give further evidence that uptake blockade is not a necessary condition for potentiation of neuronal responses to the monoamines.

We have previously reported that the hallucinogenic monoamine mescaline (3,4,5-trimethoxyphenylethylamine), applied by microelectrophoresis, can evoke responses in cortical neurones which are similar to those evoked by noradrenaline (Bevan, Bradshaw, Roberts & Szabadi, 1974). It is known that mescaline has an extremely low affinity for uptake mechanisms in the periphery (Iversen, 1967). Moreover, it has recently been reported that although mescaline is accumulated into cortical synaptosomes by an active process, this uptake is not affected by desipramine (Bevan, 1975). In the present study we examined whether neuronal responses to mescaline can be potentiated by desipramine.

Methods Single spontaneously-active neurones were studied in the cerebral cortices of cats and rats anaesthetized with halothane. All the drugs were applied by microelectrophoresis. Our techniques for the surgical preparation of the animals, for the extracellular recording of action potentials and the microelectrophoretic application of drugs from six-barrelled micropipettes have been described elsewhere (Bradshaw *et al.*, 1974; Bevan *et al.*, 1974; 1975a; 1975b). The micropipettes contained the following drug solutions: 4 M NaCl (two barrels: one for recording, one for current balancing); 0.05 M mescaline hydrochloride (pH 4.0); 0.05 M noradrenaline bitartrate (pH 3.5); 0.05 M sodium glutamate (pH adjusted to 8.5 with NaOH); 0.15 M desipramine hydrochloride (pH 4.5). Repeated responses to mescaline were compared before and after a brief application of desipramine (25–100 nA; 20–60 seconds). (Cells were excluded if the variation between control responses exceeded ± 10%; Bradshaw *et al.*, 1974). Only excitatory responses were selected for study in these experiments.

Results The effect of desipramine was studied in the cat on 11 cortical neurones which responded with a clear excitation to mescaline. On 5 cells the response to mescaline was potentiated following an application of desipramine. (A response was regarded as potentiated if there was an increase of 20% or more over the size of the mean control response; Bradshaw *et al.*, 1974). On 6 cells desipramine antagonized the response to mescaline. (A response was regarded as antagonized if there was a decrease of 20% or more in the size of the response compared with the mean of the control responses; Bradshaw *et al.*, 1974). On 2 cells both antagonism and potentiation could be observed following a brief application of desipramine: the antagonism preceded the potentiation. An example of a study in which both potentiation and antagonism could be observed on the same cell is shown in Figure 1a and b.

Seventeen cortical neurones were studied in the rat.

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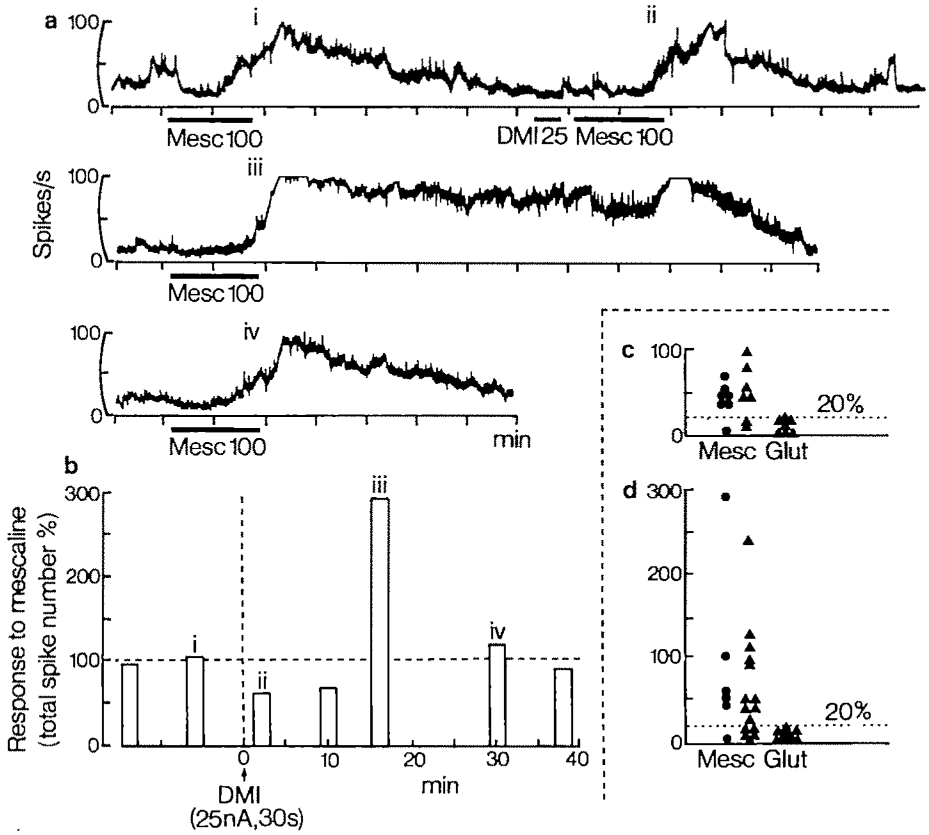

Figure 1 (a and b) Effect of desipramine on responses to mescaline of a single neurone in cat cerebral cortex. (a) Excerpts from the ratemeter recording of the firing rate of the neurone. Horizontal bars indicate drug applications; numbers refer to the intensity of the ejecting current (nA). (b) Time-course of entire study. Height of columns indicate the total number of action potentials generated in response to each application of mescaline, expressed as % of mean control (see Bradshaw *et al.,* 1974). Numbers above columns indicate sections of the study shown in the excerpts above. Following a brief application of desipramine the response to mescaline was first antagonized and later potentiated. (c and d) Summary of results obtained on all cells. (c) Antagonism: each point shows the maximum degree of antagonism observed in one individual cell (e.g. response ii in b). (d) Potentiation: each point shows the maximum potentiation observed in one individual cell (e.g. response iii in b). Closed circles refer to results obtained in cats, closed triangles to results obtained in rats.

In these studies, glutamate was used as a control agonist. Desipramine potentiated the response to mescaline on 10 cells; on 5 of these the potentiation was preceded by antagonism of the response. Responses to glutamate were not affected by desipramine.

The maximum degrees of antagonism and potentiation seen in each cell are shown in Figure 1c and d respectively.

Discussion The effects of desipramine on responses to mescaline are similar to its effects on responses to noradrenaline (Bradshaw *et al.,* 1974; Bevan *et al.,* 1974). Both antagonism and potentiation of neuronal responses to mescaline could be observed. The antagonism may reflect the α-adrenolytic action of desipramine (Callingham, 1967), since mescaline and noradrenaline probably activate similar receptors on cortical neurones (Bevan *et al.,* 1974). The potentiation of responses to mescaline is more difficult to explain: uptake mechanisms are unlikely to be involved, since desipramine does not block the uptake of mescaline into cortical synaptosomes (Bevan, 1975). An alternative possibility is that the potentiation has a post-synaptic origin: desipramine may block 'masked' inhibitory receptors on the post-synaptic membrane and thus increase the size of the observed excitatory response to mescaline (Bradshaw *et al.,* 1974; Bevan *et al.,* 1974).

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CALCITONIN: ANTAGONISM AT INTESTINAL MUSCARINIC RECEPTORS

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The action of calcitonin was studied on the motility of isolated innervated segments of rabbit and guinea-pig intestines as well as longitudinal muscle with adherent myenteric plexus dissected from the guinea-pig ileum. Calcitonin (0.25 µM) antagonized contractile responses to acetylcholine and the cholinergic response to electrical field stimulation. This hormonal effect was relatively specific since it was not observed at nicotinic receptors or adrenoceptors, nor did calcitonin act as a local anaesthetic or directly on the contractile machinery of smooth muscle. Perivascular adrenergic and intrinsic non-adrenergic inhibitory responses also were unaffected by calcitonin. However, calcitonin did have antihistaminic properties directed against H_1-receptors. The concentration of calcitonin required to achieve muscarinic antagonism in our experiments is not reached at the resting level of circulating hormone.

Introduction Calcitonin lowers the calcium concentration of blood primarily by inhibition of bone resorption, but the hormone also inhibits renal tubular calcium reabsorption and intestinal calcium absorption (Haymovits & Rosen, 1972). Recent studies have indicated that calcitonin may have additional effects on gastrointestinal function besides inhibition of calcium absorption. For example, in the cat and in man, calcitonin decreases basal, gastrin, and histamine-stimulated gastric acid secretion (Hesch, Hufner, Hasenjager & Creutzfeldt, 1971; Becker, Konturek, Reeder & Thompson, 1973; Becker, Reeder, Scurry & Thompson, 1974). There is also evidence that calcitonin decreases release of gastrin (Becker *et al.*, 1973; Becker *et al.*, 1974) and inhibits exocrine secretion of pancreatic enzymes (Schmidt, Hesch, Hufner, Paschen & Creutzfeldt, 1971). Moreover, gastrointestinal symptoms have been reported by 10% to 20% of patients receiving calcitonin for treatment of Paget's disease (Haymovits, Wright, Ling, Hobitz & Tayag, 1975). These additional effects on secretion, and perhaps the symptoms associated with calcitonin therapy, could be explained if calcitonin either antagonized the release or the action of acetylcholine and histamine. In order to test the involvement of acetylcholine in the gastrointestinal actions of calcitonin, the effects of the hormone on the motility of innervated intestinal smooth muscle preparations was studied *in vitro*.

Methods Adult male Hartley guinea-pigs and New Zealand rabbits were used. Two preparations were obtained from guinea-pigs, a segment of intact ileum, and a segment of isolated longitudinal muscle with adherent myenteric plexus (Paton & Zar, 1968). Rabbit ileum was mounted to permit transmural (Paton, 1955) or perivascular nerve stimulation (Finkelman, 1930). All preparations were studied in an organ bath containing oxygenated Krebs solution at 37°C and mechanical activity was recorded isotonically. Intact ileum was stimulated transmurally and the longitudinal muscle-myenteric plexus preparation was stimulated between two stainless steel wire mesh electrodes positioned on either side of the tissue. Rectangular pulses each of less than 0.2 ms duration were used to deliver supramaximal stimuli. These pulses activate nerve but not smooth muscle.

Concentrations of drugs given in the text refer to the final concentration in the bath. The drugs used were: acetylcholine iodide, hyoscine hydrobromide, histamine diphosphate, nicotine tartrate, and (—)-noradrenaline bitartrate. Either purified human calcitonin (Haymovits & Levin, 1975) prepared by solid phase peptide synthesis, and standardized against pure synthetic calcitonin obtained from CIBA Pharmaceutical Co. (Sieber, Brugger, Kamber, Riniker & Riettel, 1968), or the CIBA product was used. Studies performed with either preparation yielded identical results. Stock solutions of calcitonin contained 5% albumin. The diluent alone had no effect on any of the preparations studied.

Results The action of calcitonin (0.25 µM) on the activity of the intact rabbit and guinea-pig intestine is shown in Figure 1. The rabbit intestine shows

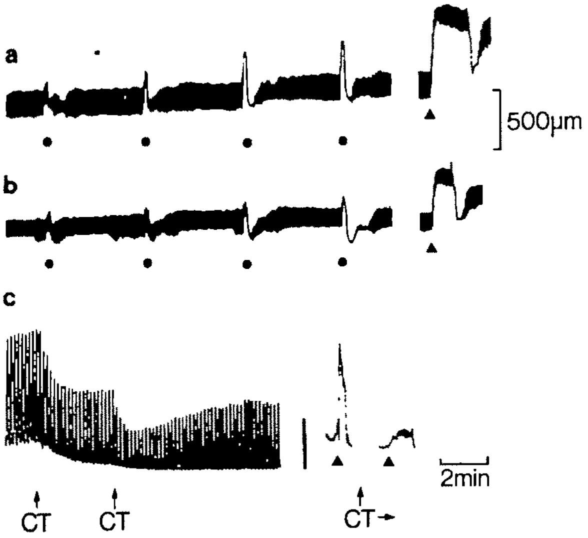

Figure 1 Effects of calcitonin on spontaneous and electrically stimulated intestinal motility *in vitro*. (a) and (b) Rabbit ileum stimulated transmurally at the dots for 10 s at successively 5, 10, 20 and 30 Hz: (a) control, (b) in presence of calcitonin (0.25 μM); (▲) acetylcholine (0.37 μM). (c) Guinea-pig ileum, each contraction is the response to a single shock. Calcitonin (CT) was added twice, first to give a concentration of 0.25 μM, then 0.5 μM. Acetylcholine (▲, 3.7 nM) was tested in the presence and absence of calcitonin (0.25 μM). Similar records were obtained from intact ileum and isolated longitudinal muscle-myenteric plexus.

pendular spontaneous activity and responds to trains of transmural electrical stimuli with a biphasic response consisting of an initial contraction and, particularly at higher frequencies of stimulation, with an after-relaxation (Figure 1a). The spontaneous activity is known to be myogenic (Gershon, 1967). Both phases of the response to transmural stimulation are known to be neurogenic (Paton, 1955; Gershon, 1967). The excitatory component of the response is mediated by cholinergic neurones and the relaxant response is mediated by non-adrenergic intrinsic inhibitory neurones (Gershon, 1967; Paton & Zar, 1968; Burnstock, 1972). The guinea-pig intestine shows no spontaneous activity but responds with a contraction to single shocks delivered transmurally (Figure 1c). Calcitonin does not inhibit the myogenic pendular activity of the rabbit intestine although, like hyoscine, it decreases slightly the amplitude of the pendular movements. On the other hand, calcitonin antagonizes the contractile phase of the response to transmural stimulation while, particularly at higher frequencies of stimulation, augmenting the relaxant component (compare Figure 1a and b). The contractions of the guinea-pig ileum to transmural shocks are also inhibited by calcitonin (Figure 1c). Similarly, in both preparations the contractions of the tissue in response to the addition of acetylcholine (3.66 nM to 3.66 μM) are antagonized by calcitonin (Figure 1, right). The lowest concentration at which an effect of calcitonin could be detected consistently was 10 nM.

In order to determine whether the action of calcitonin on intestinal motility is limited to antagonism of the muscarinic action of acetylcholine, the effects of calcitonin on relaxant responses of the rabbit ileum to stimulation of adrenergic and non-adrenergic inhibitory nerves as well as addition of noradrenaline and nicotine were also studied. These experiments were all carried out in the presence of hyoscine (0.23 μM). The rabbit intestine was used because its high tone and spontaneous activity permit relaxant responses to be seen. Calcitonin (as much as 2.5 μM) has no effect on the relaxation of this preparation to noradrenaline (0.31 μM) or nicotine (20 μM). The relaxant response to nicotine is mediated by non-adrenergic intrinsic inhibitory neurones (Gershon, 1967; Burnstock, 1972). This suggests that calcitonin does not directly relax the smooth muscle, and does not act as an antagonist at adrenoceptors or nicotinic receptors. This suggestion is confirmed by the further observation that calcitonin also fails to affect responses to stimulation of the perivascular adrenergic nerves or transmural stimulation of the intrinsic non-adrenergic inhibitory nerves. These experiments also rule out the possibility that calcitonin could be acting as a local anaesthetic.

KCl (5.4 mM and 54 mM) was added to depolarize smooth muscle directly and induce myogenic contractions. Hyoscine (0.23 μM) was present in order to eliminate any possibility of a cholinergic component to the contraction. The amplitude of the response to either concentration of KCl (1.1 ± 0.2 mm; 7.7 ± 0.5 mm; mean ± s.e.) is not significantly affected by calcitonin (2.5 μM) (1.0 ± 0.1 mm, 8.4 ± 0.4 mm). Therefore, calcitonin does not appear to influence the contractile properties of smooth muscle directly.

The contractile response of the guinea-pig ileum to histamine is mediated by H_1-receptors on smooth muscle and does not have an indirect cholinergic component (Gershon, 1967; Burnstock, 1972). The concentration-effect curve to histamine (6.5 nM to 33 μM) was shifted to the right by calcitonin (2.5 μM). This suggests that calcitonin also has antihistaminic properties directed against the H_1-receptors of intestinal smooth muscle.

Discussion The results obtained in these experiments indicate that calcitonin has a weak anticholinergic effect. This antagonism, exerted at the muscarinic receptors for acetylcholine is adequate to account for almost all of the observed effects of calcitonin on isolated innervated smooth muscle preparations. Thus, cholinergic excitatory responses to transmural electrical stimulation are diminished in amplitude and simultaneous non-cholinergic inhibitory responses are unmasked by calcitonin and appear relatively augmented. This antimuscarinic effect is relatively specific. Calcitonin has no direct action on smooth muscle contraction, and is not an antagonist at nicotinic receptors for acetylcholine or at adrenoceptors. Moreover, the hormone does not act as a local anaesthetic nor does it affect adrenergic or non-adrenergic inhibitory neurotransmission. However, calcitonin is not entirely specific in its action. The hormone also has antihistaminic properties. The possibility that calcitonin might decrease release of acetylcholine was not studied because it was not necessary to postulate this additional action to explain the effects of the hormone.

It seems reasonable to assume that the atropine-like antimuscarinic effect of calcitonin may account for the actions of the hormone on glandular secretion which were listed previously. All of these are under at least partial cholinergic control (Grossman, 1970; Hirschowitz, 1975). However, it is not likely that calcitonin exerts these effects at the concentrations at which it normally circulates in blood (about 50 pM to 250 pM: Deftos, Goodman, Engelman & Potts, 1971). Gastrointestinal actions might accompany administration of calcitonin in pharmacological doses for therapeutic purposes or its autonomous hypersecretion by medullary carcinoma of the thyroid (Gautvik & Tashjian, 1974).

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EFFECTS OF SYMPATHOMIMETIC AMINES ON ^{45}Ca EFFLUX FROM LIVER SLICES

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The efflux of ^{45}Ca from slices of guinea-pig and rabbit liver is greatly increased by α-adrenoceptor agonists. Isoprenaline is much less effective. The effects of these agents on the efflux of ^{45}Ca mirror their actions on ^{42}K loss and suggest that the two may be related. Glucose release from both rabbit and guinea-pig liver slices is increased to a similar extent by either α- or β-receptor agonists. The possible relationship between Ca and K movements and the production of glucose is discussed.

Introduction Previous work (Haylett & Jenkinson, 1972a, b) has shown that activation of an α-like receptor causes a rise in the potassium permeability of guinea-pig liver cells. This is reflected by an increase in the rate coefficient for ^{42}K efflux, a net loss of cell potassium, increased membrane conductance and hyperpolarization of the cells. The same study also rather unexpectedly showed that α- as well as β-adrenoceptor agonists increased glucose release from guinea-pig liver. The β-mediated response almost certainly involves an increase in the activity of adenylate cyclase. However, α–receptors are not thought to activate this enzyme. In keeping with this, recent work in this laboratory (Osborn, 1975) has shown that α-agonists (in contrast to β-agonists) do not cause a significant rise in cyclic adenosine-3′,5′-monophosphate content of guinea-pig liver slices. An alternative possibility is that the α-mediated increase in glucose release results from changes in intracellular ion concentrations. It has been shown in skeletal muscle that phosphorylase b kinase, an important enzyme in the glycogenolytic pathway, can be activated by low concentrations of Ca (Heilmeyer, Meyer, Haschke & Fisher, 1970) and it seemed possible that α-receptor activation might increase the breakdown of hepatic glycogen in a similar way. It thus seemed worthwhile to study the actions of sympathomimetic amines on calcium movements in this tissue.

Methods The experiments were performed on slices cut from the liver of either male guinea-pigs or rabbits. Details of the slicing procedure and information on the condition of the slices may be found in Haylett & Jenkinson (1972a). The slices were incubated at 38°C in a medium containing (mM): NaCl 125, KCl 6, $CaCl_2$ 1, $MgSO_4$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 15, Na pyruvate 2 (pH 7.4 when gassed with 5% CO_2 in O_2) and were loaded with ^{42}K and ^{45}Ca for the period from 90 to 180 min after their preparation. Efflux of the isotopes and release of glucose were measured by passing the slices through a series of test-tubes containing 5 ml of non-radioactive solution at 2 min intervals. ^{42}K released from the slices was measured by Cerenkov counting of 2 ml aliquots of the washout solutions, and ^{45}Ca in the same samples was counted by adding scintillation fluid after ^{42}K had decayed. The slices were weighed and sonicated in incubation medium at the end of the experiment and samples of the suspension taken for counting. Glucose in the washout fluid was analysed by the simple colorimetric method, based on the reducing property of glucose, described previously (Haylett & Jenkinson, 1972b).

Results Figure 1 shows the effects of (–)-noradrenaline (1 μM), (–)-amidephrine (10 μM) and (–)-isoprenaline (1 μM) on ^{45}Ca, ^{42}K and glucose release from rabbit liver slices. Amidephrine is a selective α-adrenoceptor agonist (Dungan, Stanton & Lish, 1965) and isoprenaline at this concentration will have little effect on α-receptors. It can be seen that noradrenaline and amidephrine greatly increase the loss of ^{42}K, ^{45}Ca and glucose whereas isoprenaline produces a substantial change in glucose release alone. The effect of isoprenaline on ^{42}K and ^{45}Ca loss was rather variable, and sometimes undetectable. Taken together, the results suggest that α-receptors can elicit all three responses and that β-receptors, whilst consistently causing release of glucose, have a variable and generally small effect on ion movement.

Similar experiments with guinea-pig liver slices showed that (–)-amidephrine (10 μM) again caused a substantial increase (300–400%) in the rate of ^{45}Ca efflux whereas (–)-isoprenaline (50 nM, maximal for glucose release from guinea-pig liver) had a very small effect (increased by about 15%).

Examination of the ^{45}Ca efflux curves for guinea-pig liver slices indicated that at the time of drug application (35 min after removal from the ^{45}Ca solution) a substantial part of the accumulated ^{45}Ca has already been lost, mainly from a rapidly exchanging fraction which probably includes tracer in the extracellular space and perhaps also some bound to damaged tissue at the surface of the slices. The

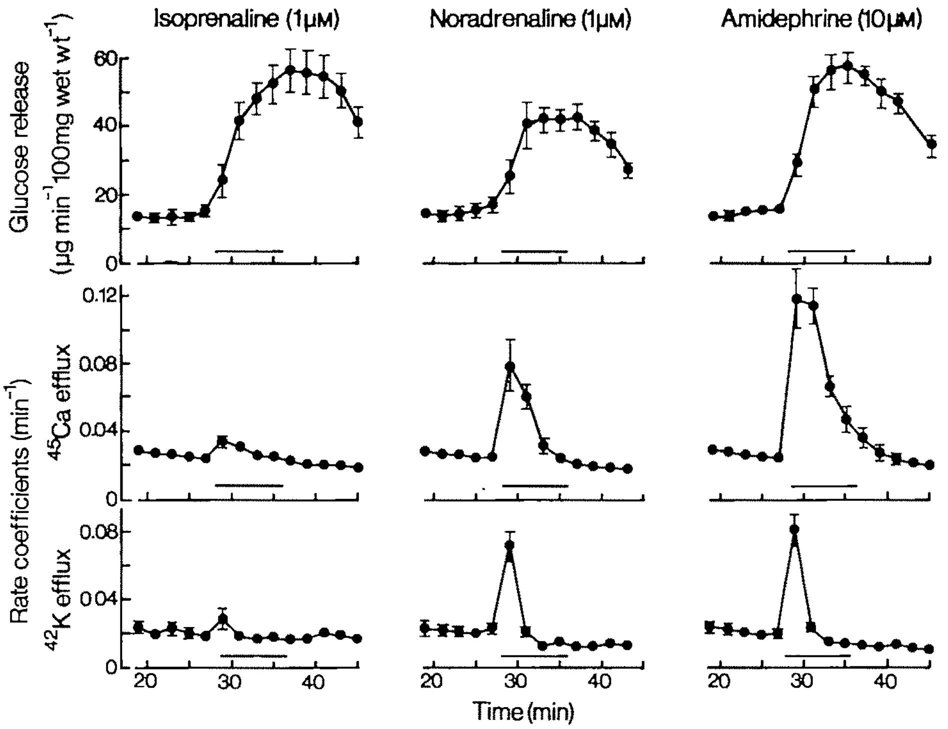

Figure 1 Effects of (–)-isoprenaline (1 μM), (–)-noradrenaline (1 μM) and (–)-amidephrine (10 μM) on the simultaneously measured release of glucose, ^{45}Ca and ^{42}K from rabbit liver slices. Drugs were applied for 4 successive 2 min periods as indicated by the horizontal bars. Each point is the mean of 3 or 4 observations and s.e. means are indicated where they exceed the size of the symbol.

remaining efflux appears to be coming from tissue compartments containing less than 1 mmol Ca/kg wet wt. as compared with a total Ca content of 2.6 ± 0.1 (s.e., $n=22$) (K. Koller, personal communication). However, the fact that α-receptor activation can cause the rapid loss (within a few minutes) of more than 30% of this residual ^{45}Ca suggests that substantial changes in calcium distribution are occurring.

Discussion The main new finding is that α-receptor activation can greatly increase the efflux of ^{45}Ca from both rabbit and guinea-pig liver slices. The mechanism for this loss is as yet uncertain; active extrusion of calcium from the cells, release from internal binding sites (in particular mitochondria) and increased membrane permeability to Ca all being possible. The last two would probably lead a to a rise in the free concentration of Ca, $[\mathrm{Ca}]_i$, within the cell. As already suggested this could lead to the activation of glycogenolytic enzymes. It is also possible that the effect on cell membrane potassium permeability may be the result of a rise in $[\mathrm{Ca}]_i$. Experiments by Romero & Whittam (1971) suggested that the permeability of the erythrocyte membrane to potassium could be increased by raising $[\mathrm{Ca}]_i$ and recent support for this has come from studies with the Ca-ionophore, A23187, which causes a Ca-dependent loss of K from red cells (Reed, 1973) and from salivary glands (Selinger, Eimerl & Schramm, 1974). Furthermore, Meech (1974) has shown that injection of Ca directly into snail ganglion cells leads to an increase in K permeability and hyperpolarization.

In conclusion it is tempting to propose that α-receptor activation in guinea-pig or rabbit liver leads to a rise in $[\mathrm{Ca}]_i$ which then brings about the three actions examined in the present work; increased loss of ^{45}Ca, ^{42}K and glucose.

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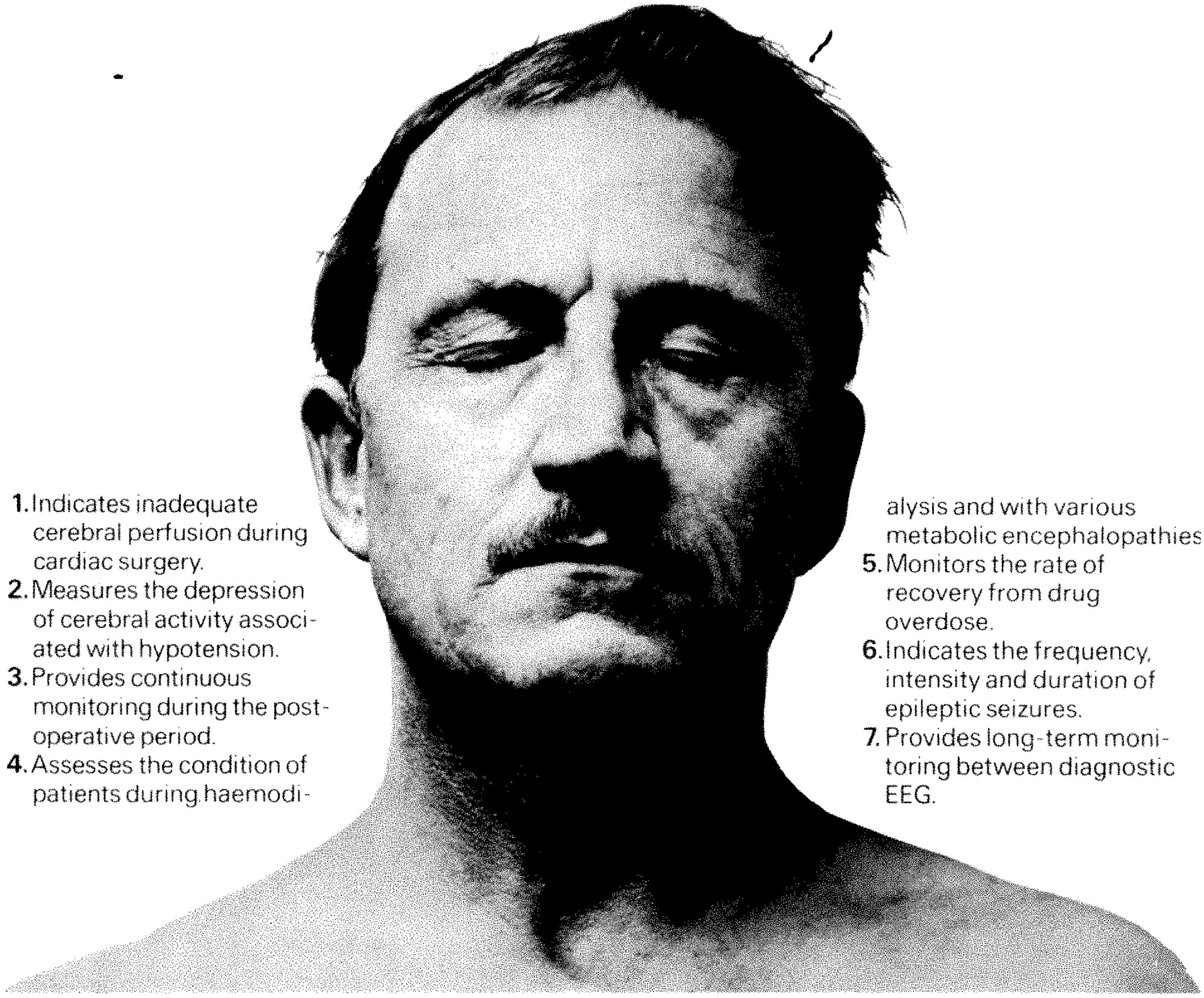

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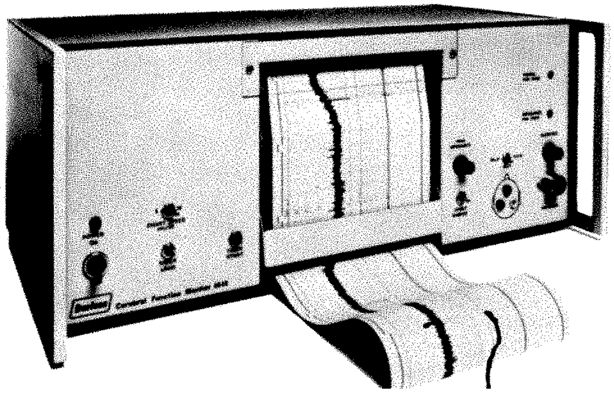

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THE EFFECT OF QUINIDINE ON MEMBRANE ELECTRICAL ACTIVITY IN FROG AURICULAR FIBRES STUDIED BY CURRENT AND VOLTAGE CLAMP

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1 The action of quinidine sulphate 50 μM has been investigated on frog auricular trabeculae transmembrane currents recorded with a double sucrose gap apparatus. Results were obtained either in current or in voltage clamp conditions.

2 Quinidine modified the time course of repetitive activity elicited by long lasting depolarizing currents and reduced the range of current over which repetitive activity could be triggered, eventually abolishing repetitive responses altogether.

3 Several authors have emphasized the limitations of the voltage clamp method. Taking into account these limitations, the numerical values of the parameters obtained in the present work must not be considered as exact values but may be interpreted as indicators of the variations of the parameters.

4 The results are in agreement with previous findings that the main features of the action of quinidine are to produce (a) a reduced maximum rate of depolarization (MRD), (b) a reduced total amplitude of action potential; (c) a flattening of the plateau of the action potential, (d) a slight prolongation of the tail of the action potential, (e) an increased effective refractory period without greatly prolonging action potential duration, (f) no change of resting potential and of 50% repolarization time.

5 The analysis of ionic conductances has provided explanations for the above effects.

6 Quinidine reduced the reactivation kinetics of the sodium inward current, and decreased sodium conductance and the steady state of activation. These effects account for (a) and (b).

7 Quinidine increased the activation and inactivation time constants of sodium conductances, which account in part for (e).

8 Quinidine delayed reactivation of slow inward current, reduced calcium conductance, and decreased the steady state of activation of calcium conductance. These effects could account for (c).

9 The amplitudes of the two components of the delayed conductances responsible for repolarization were decreased by quinidine, and the time constant of activation for the faster of the two was slowed. These effects could account for (d) and in part for (e).

Introduction

Microelectrode intracellular recordings have permitted a decisive advance in the electrophysiological study of the mode of action of antiarrhythmic drugs. It has been demonstrated that quinidine does not alter the resting potential (Vaughan Williams, 1958); that the intracellular concentrations of potassium and sodium remain unchanged during the action of quinidine (Goodford & Vaughan Williams, 1962); that this drug strongly decreases the maximum rate of rise (Weidmann, 1955) and that it lengthens the effective refractory period even if the action potential duration remains the same (Vaughan Williams, 1961). From the above evidence, Vaughan Williams (1958) concluded that quinidine has a direct effect on the cardiac membrane. Therefore it was of interest to study the action of quinidine with the double sucrose gap technique which permits the measurement of the transmembrane ionic conductances responsible for cardiac electrical activity. This technique has previously been applied to frog auricular trabeculae by Rougier, Ildefonse & Gargouïl (1966), to the rat ventricular myocardium by Besseau & Gargouïl (1969) and to monkey intraventricular fibres by Walden, Kréher, Aka & Tricoche (1973).

Owing to the fact that a certain loss of voltage control exists when voltage clamping the preparation (Johnson & Lieberman, 1971; Tarr & Trank, 1974; Léoty & Poindessault, 1974; Ramón, Anderson, Joyner & Moore, 1975), the results obtained from the experiments described here must be considered as

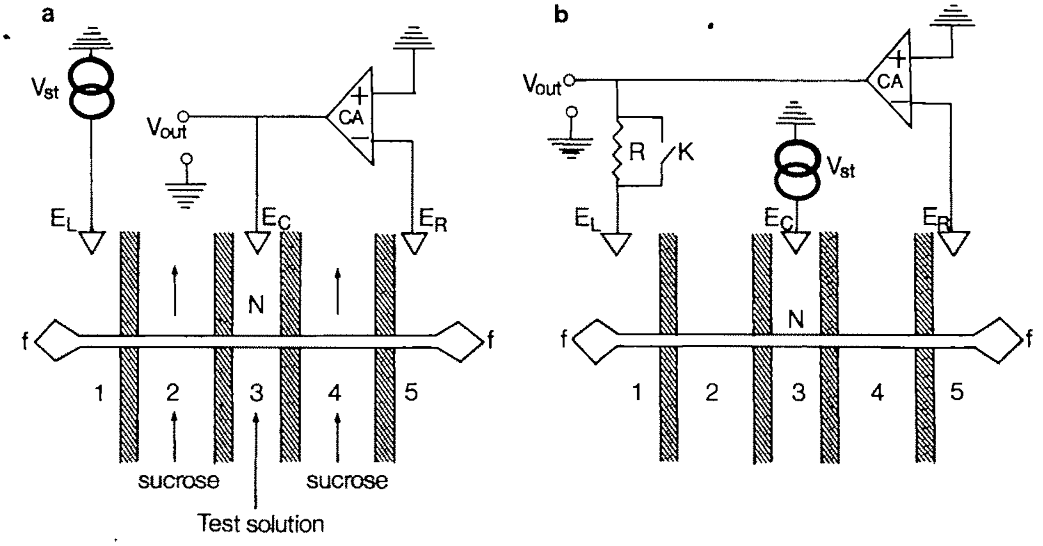

Figure 1 Arrangement for current and voltage clamp recordings. (a) Stimulation and recording diagram in current clamp conditions. E_R is the reference electrode used to hold the intracellular potential of the artificial node N; E_L is the left electrode or electrode for control of the transmembrane current in the artificial node N. (b) Stimulation and recording diagram in voltage clamp conditions. E_R has the same role as in (a); E_c becomes the electrode for control of the transmembrane potential in the artificial node; E_L becomes the electrode measuring the transmembrane current in the artificial node. The different compartments are bathed as follows: 1 and 5 by Ringer solution, 2 and 4 by sucrose solution which is used to insulate the central artificial node from compartments 1 and 5 (insulation being also provided by means of partitions made of mixed vaseline and paraffin between each compartment); 3 or test-compartment by Ringer solution or modified Ringer solution (single or with the studied drug added). *ff* is the cardiac auricular preparation transversely arranged on the different compartments; V_{st} is the stimulating signal, V_{out} is the recorded signal and CA is the control amplifier.

qualitative results which reveal only the direction of variation of the different parameters during quinidine's action. Nevertheless it must be pointed out that the limitations of the voltage clamp experiments are largely dependent on the type of double sucrose gap device used and from recent work by de Hemptinne (unpublished observations) that the partitions with vaseline seals lead to less important errors than a double sucrose gap technique using fluid partitions. So the technique used in the present work can be considered at present as a relatively efficient one.

In this work, the phenomenological equations proposed by Hodgkin & Huxley (1952c) will be used to describe the effects of quinidine on the transmembrane conductances and their parameters.

Methods

The experiments were performed on auricular trabeculae of the frog (*Rana esculenta*) about 100 to 250 μm in diameter and about 3 to 4 mm in length. The chamber was similar to that described by Rougier, Vassort & Stämpfli (1968). The sucrose compartments were 250 μm wide and the central 'node' was 150 μm wide. The separation of solutions was made with vaseline seals. Vaseline was added to paraffin wax in such a proportion as to obtain the required viscosity at 18°C to build strong enough seals. All the electrodes were calomel half cells. Their impedance was essentially resistive (less than 50 Ω) and the junction potential difference between any of them was less than 1 mV.

The electronic circuitry

Referring to Figure 1, the voltage sensing electrode (E_R) and the current passing one (E_L) were placed in the outer (respectively right and left) compartments of the chamber. The electrode in the central compartment is placed downstream, 1 cm distant from the central node. The central compartment widens rapidly on both sides of the central node in order to lower the bath resistance. Assuming that the vaseline seals and the sucrose fluxes provide a perfect electrical isolation between the compartments i.e. no current can flow between two adjoining compartments outside the preparation, the operation of the electronic set up may be described as follows:

Current clamp conditions (Figure 1a). A stimulating voltage (V_{st}) is applied to the current pool electrode (E_L) and is converted in the intracellular longitudinal resistance between the current pool and the test node into a stimulating current. This current flows across the preparation and the central node towards electrode E_C since the inverting input of the control amplifier (CA) draws a negligible current (10^{-12} A). The potential recorded at the output of the CA is that lying between E_C and E_R. The CA clamps E_R at the earth potential since its non-inverting input is grounded. As practically no current flows in the intracellular longitudinal resistance between the central node and the voltage pool (E_R), the inside of the preparation in the central node is clamped at earth potential. Thus, the measured potential (V_{out}) represents the voltage across the preparation in the central node.

Voltage clamp conditions (Figure 1b). The aim of this circuit is to maintain a known and fixed potential across the preparation in the test node. As in the preceding description, the inside of the preparation is clamped at earth potential. The stimulating or clamp voltage is applied to E_C, so the voltage across the preparation would be that potential (V_{st}). The current required to clamp the inside of the preparation at earth potential is delivered through the current pool electrode E_L by CA. It develops the output potential (V_{out}) on the intracellular longitudinal resistance between the current pool and the test node. This current is also the current flowing across the preparation in the test node since the inverting input of the CA draws a negligible current (10^{-12} A). So the output voltage (V_{out}) of the CA is directly proportional to the current flowing in that condition out of the preparation in the test node i.e. the 'membrane' current. The longitudinal intracellular resistance between the current pool (E_L) and the test node is calculated from the variation in V_{out} when a known resistance (R) is added in series with E_L whereas the stimulation V_{st} , and thus the current, remain the same.

Solutions and experimental conditions

Quinidine sulphate (Merck) or dihydroquinidine gluconate (Houdé) 50 µM was added to a Ringer solution, which had the following composition (mM): NaCl 110, KCl 2.5, $CaCl_2$ 1.8 and Tris(hydroxymethyl) aminomethane 10. The pH was adjusted to 7.8 with HCl. Two permeability inhibitors were used in an attempt to obtain the net membrane currents: tetrodotoxin (TTX), considered as a specific inhibitor of the fast sodium inward current, was used at 0.1 µg/ml. Manganese chloride, 2 mM, which is considered to inhibit the slow inward current, was also used. The use of manganese did not always produce a total suppression of the slow inward current and results of these experiments were discarded. Such inhibitors have been successfully used by Rougier, Vassort, Garnier, Gargouïl & Coraboeuf (1969). The experiments were performed at a temperature near 18°C. The rate of stimulation was adjusted so that no new stimulus was delivered before complete repolarization (in current clamp conditions) or before complete inactivation or deactivation of the currents (in voltage clamp conditions) had occurred.

Experimental protocols

Experiments carried out in current clamp conditions. The preparation was stimulated by constant currents so as to obtain an action potential (AP) whose amplitude, duration and rate of rise were used to decide if the experiment was worth pursuing. The stability of the AP or repetitive activity (RA) was then checked. The test compartment was bathed with a Ringer solution added with the drug under study and recordings were subsequently made at different times. After that, the preparation was again bathed with Ringer solution (to study the reversibility of the drug effect). The depolarizing stimulating currents were displayed on the oscilloscope screen as variations of potential, so it was then necessary, in voltage clamp conditions, to measure the internal longitudinal resistance of the preparation in order to calculate in amperes the amplitude of the depolarizing currents used for stimulation (see appendix). For AP time course studies, the rate of stimulation, chosen in order to obtain a constant AP time course, was approximately one stimulus every 10 seconds.

Experiments carried out in voltage clamp conditions. The state of the preparation bathed in a Ringer solution was first checked by observation of the AP time course. Then the preparation was placed in voltage clamp conditions. The type of study to be tried was decided from observation of the time course and amplitude of the studied current and from the determination of its reversal potential.

Studies concerning the inward currents. The preparation was held at a potential allowing the current to be fully activated when the preparation was stimulated with a suitable value of depolarizing pulse. The test compartment was then bathed with a Ringer solution with added TTX (if slow inward current was studied) or with $MnCl_2$ (if fast inward current was studied) or with a modified Ringer solution allowing only the calcium inward current to develop. By using $MnCl_2$ it was possible partly to reduce the artifact appearing on the rectangular time course of the imposed potential due to the presence of the slow inward calcium-sodium current. The recordings obtained in such conditions were taken as the reference ones. Then the internal longitudinal resistance of the preparation was measured. The test compartment was then bathed with the same solution as above but with drug added and the same recordings and measurements were made after the drug effect was obtained. It was possible to use this general procedure to obtain the current-voltage curves, the reactivation curves and the inactivation curves. Nevertheless, current-voltage curves as current time courses used to calculate the conductances time courses were also obtained by the subtraction methods described below. The subtractions were made over the range of clamp potentials allowing inward currents to develop, these inward currents being the fast sodium current and the slow calcium-sodium one. In some experiments a series of four solutions were used: (1) a solution inhibiting both inward currents; (2) a solution inhibiting only one inward current; (3) the effect of quinidine on one inward current; (4) a solution inhibiting both inward currents. Subtraction of the current in (1) from (2) gave the control or reference size of the current under study while subtraction of the current in (4) from (3) gave its size in the presence of quinidine. This type of protocol allowed relatively easy subtractions but its major disadvantage was the long time needed to perform it, such a long time decreasing the probability of success in the experiments. In most experiments a series of three solutions were used: (1) a solution allowing only the studied current to develop; (2) the effect of quinidine on this current; (3) a solution inhibiting the inward currents. Subtraction of the current in (3) from (1) gave the control or reference size of the current under study while subtraction of the current in (3) from (2) gave its size in the presence of quinidine. This type of protocol is simpler and needed less time to perform but it needed very good stability of the preparation during the series of three solutions.

Studies concerning the delayed outward currents. The test compartment was bathed with a Ringer solution with TTX and $MnCl_2$ added. When the inward currents were entirely inhibited the reversal potential of each outward current component was determined. Recordings of the reference current were made in this first medium and then the test compartment was bathed with a Ringer solution with TTX, $MnCl_2$ and quinidine added. During the action of quinidine the same recordings as above were made. Particular care was taken concerning the stimulation rhythm because it was essential before applying another stimulation to wait for a complete deactivation of the delayed currents (see the observations of de Hemptinne, 1971).

Nomenclature

TRC is the triggering range of currents, that is the range of current by which a repetitive activity can be triggered. V (mV) is the variation of the membrane potential from the resting potential $E_m = -70$ to -75 mV (Rougier *et al.*, 1968) being taken as zero. Depolarizations are positive values of V and negative values are hyperpolarizations. V_{Na}, V_{Ca}, V_{x1}, V_{x2} are respectively the reversal potentials for sodium and calcium inward currents, and for the fast and slow components of the delayed outward currents. τ_{re1} and τ_{re2} are the reactivation time constants of a given inward current (respectively for the fast component and the slow one). I_{Na}, I_{Ca}, I_{Ca-Na}, $I_t = \Sigma I = i_{x1} + i_{x2}$ are respectively the fast sodium current, the slow calcium, the slow calcium-sodium and the total delayed outward currents. g_{Na}, g_{Ca}, $\Sigma g = g_{x1} + g_{x2}$ are respectively the conductances calculated from the same currents as above. The nomenclature used for i_{x1} and i_{x2} corresponds to systems 1 and 2 of Brown & Noble (1969a, b) and is identical to that used by Besseau (1972), as regards i_{x1}, i_{x2}, g_{x1} and g_{x2}. i'_{x1} and i'_{x2} are the values of the tail currents determined at the origin on semi-logarithmic plots (i'_{x1} for the fast component and i'_{x2} for the slow component of the delayed current).

Conductance parameters for g_{Na}. m_∞ is the steady state of activation function; 'a' is the exponent of the activation variable m; g'_{Na} is a quantity proportional to the steady state of the activation (the exponent of m being known); $1/\tau_m$ is the reciprocal activation time constant; $1/\tau_h$ is the reciprocal inactivation time constant; α_m and β_m are the rate constants of the activation system; h_∞ is the steady state of inactivation function.

Conductance parameters for g_{Ca}. In order to be homogeneous with the nomenclature used by Reuter (1974) the symbol *m* is replaced by *d* and the symbol *h* is replaced by *f*; consequently, the parameters are d_∞, f_∞, 'a', g'_{Ca}, $1/\tau_d$, $1/\tau_f$, α_d and β_d.

Conductance parameters for g_{x1} *and* g_{x2}. $n_{\infty 1}$ and $n_{\infty 2}$ are the steady states of the activation functions; $1/\tau_{n1}$ and $1/\tau_{n2}$ are reciprocal activation time constants; α_{n1}, β_{n1} and α_{n2}, β_{n2} are the rate constants.

Calculations

The details of the different calculations are explained in the appendix. The statistical significance of differences between means was calculated from Student's t test.

Results

Current clamp conditions

Action potential time course. Figure 2(a) shows an AP obtained in a Ringer solution, with an amplitude of

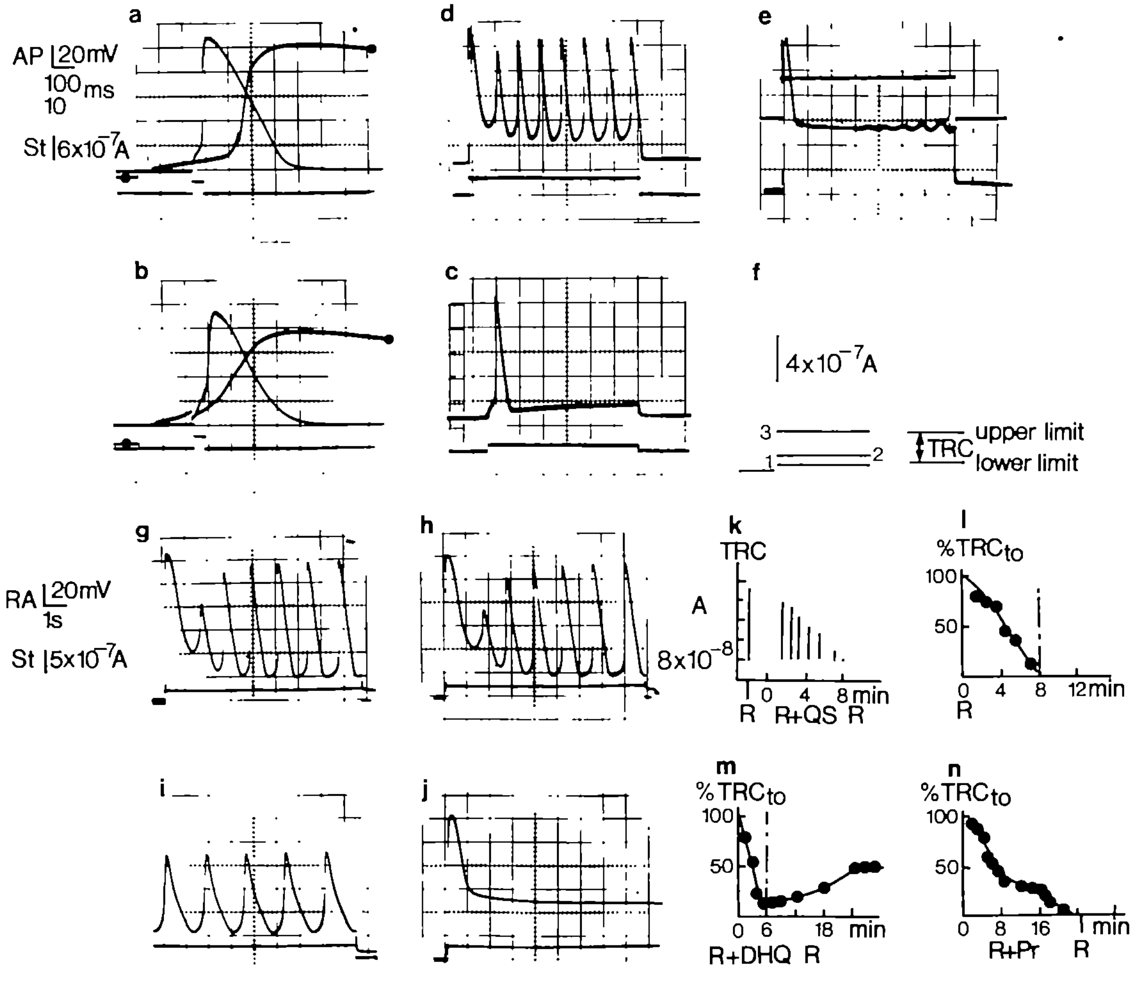

Figure 2 The effect of quinidine sulphate 50 μM on the action potential time course, the repetitive activity and the triggering range of current (TRC) of this repetitive activity. (a & b) Modification of the action potential in presence of quinidine: (a) in a Ringer solution; (b) in a Ringer solution + quinidine after 24 min of drug action. The upper trace is the action potential at a sweep speed of 100 ms/division, the middle trace is the depolarization phase of the action potential at a sweep speed of 10 ms/division; the lower trace is the stimulating current. (c, d, e and f) Method of obtaining the triggering range of current (TRC): (c) potential response (upper trace) to a depolarizing current, whose amplitude is labelled 1 in (f) is only a single spike, so the amplitude of stimulation labelled 1 is lower than the lower limit of the TRC; (d) the potential response (upper trace) to a depolarizing current, whose amplitude is labelled 2 in (f) is repetitive activity, so amplitude of stimulating current labelled 2 is within the TRC; (e) the potential response (lower trace) to a depolarizing current, whose amplitude is labelled 3 in (f) is a single spike and a few oscillations at the end of the pulse, so amplitude of stimulation labelled 3 is higher than the upper limit of the TRC; (f) the TRC corresponds to values of depolarizing currents which elicited repetitive activity. (g, h, i and j) Modification of the time course of the induced repetitive activity in the presence of quinidine. Upper trace is the potential response and lower trace is the depolarizing current the amplitude of which is the same for the four figures: (g) in Ringer solution; (h) after 4 min of quinidine action; (i) after 8 min of drug action; (j) after 10 min of drug action, repetitive activity has ceased. (k, l, m and n) Modification of the TRC in presence of antiarrhythmic agents: (k) decrease of the amplitude of TRC in presence of quinidine sulphate 50 μM (QS) applied to the preparation from 0 to 8 min; the preparation was then bathed with a Ringer solution (R); (l) same experiment but the result is expressed in percentage of the TRC obtained in a Ringer medium (=100% TRC); after 8 min the preparation is again bathed in a Ringer solution; (m) decrease of the TRC in presence of dihydroquinidine 5 μM (DHQ) applied to the preparation from 0 to 6 min; then the preparation was again bathed with Ringer solution; (n) decrease of the TRC in presence of procainamide 100 μM (Pr) applied to the preparation from 0 to 25 min; after 25 min the preparation was again bathed with Ringer solution.

110 mV and maximum rate of rise of about 13.5 V/second. This low rate of rise underlines the difference between intracellular microelectrode recordings and extracellular recordings. The time for 95% of full repolarization was about 380 milliseconds. The AP time course was altered by the action of quinidine sulphate 50 μM (Figure 2b) which also induced a depression of the plateau. There was a slowing of the final repolarization phase of the AP, this slowing becoming observable after 24 min of quinidine's action; the time needed for 95% full repolarization was now 400 milliseconds. The effects of quinidine on frog atrial AP are summarized in Table 1. Quinidine induced no change in resting membrane potential: in

Table 1 Effect of quinidine sulphate 50 μM on frog atrial action potential

	No. of fibres	*AP amplitude (mV)*	*Time to 50% repolarization (ms)*	*Time to 90% repolarization (ms)*	*Time to 95% repolarization (ms)*	*Maximum rate of rise (V/s)*
Control	21	114 ∓ 1	214 ∓ 4	351 ∓ 8	390 ∓ 5	18.6 ∓ 1.2
Quinidine sulphate 50 μM	21	99 ∓ 1	204 ∓ 4	352 ∓ 7	411 ∓ 5	6.9 ∓ 0.6
Difference from control		−15	−10	+1	+21	−11.7
Statistical significance of differences from control		$P<0.001$	NS	NS	$P<0.01$	$P<0.001$

NS = not significant.

Table 2 Modification by quinidine sulphate 50 μM of the time course of repetitive activity corresponding to the experiment shown in Figure 2g, h and i

Parameters	*Control*	*Quinidine sulphate (50 μM)*	
		4 min of action	*8 min of action*
Average spike amplitude (mV) (measured from maximum diastolic repolarization level)	97	96	68
Average spike repolarization time (ms)	780	860	1230
Average spike frequency (Hz)	0.7	0.7	0.5
Average spike depolarization rate (V/s)	1	1	0.5
Average diastolic depolarization rate (V/s)	0.03	0.02	*
Difference between the potential at rest and the maximum diastolic repolarization potential during repetitive activity (mV)	17	18	21

* Not measurable.

31 experiments during the action of quinidine, the mean difference in resting membrane polarization from control values was less than 2 mV.

Induced repetitive activity. By means of weak and long-lasting depolarizing currents under current clamp conditions, it was possible to trigger repetitive activity in frog auricular fibres as in Purkinje fibres (Hauswirth, Noble & Tsien, 1969). This activity was maintained throughout the period of the depolarization (Figure 2d).

The time course of the rhythmic activity obtained in Ringer medium (Figure 2g) was modified by the action of quinidine sulphate 50 μM (Figure 2h and i). Such modifications observed in the experiment shown in Figure 2(g, h and i) are summarized in Table 2. Quinidine decreased the spike amplitude, the spike frequency, the spike depolarization rate, the diastolic depolarization rate and increased the spike repolarization time and the difference between the potential at rest and the maximum diastolic repolarization potential during repetitive activity. After 10 min of action of quinidine, in the case of the results shown in Figure 2(g, h and i) and in Table 2, all repetitive activity ceased. Table 3 shows the modification of the repetitive activity time course after 8 min of action of quinidine in 11 experiments. As regards the diastolic depolarization rate the results correspond to 4 min of action of quinidine because after 4 min the time course of the diastolic depolarization is modified in such a way that it becomes impossible to measure its slope (as in Figure 2i).

It was interesting to study the range of current which can trigger repetitive activity, the triggering range of current (TRC). The preparation was depolarized by long lasting currents (see Figure 2f). The value 1 of the depolarizing current (in Figure 2f) which corresponds to the response shown in Figure 2(c), was not strong enough to elicit repetitive activity, only a single spike being observed. If the amplitude of the depolarizing pulse was increased it became possible to elicit repetitive activity and the lowest value of depolarizing pulse which allowed repetitive activity to develop was the lower limit of the TRC. Then the amplitude of the depolarizing pulse was again increased. Value 2 (in Figure 2f) is the value of depolarization which allowed repetitive activity in Figure 2(d) to develop. Then if the amplitude of this depolarizing pulse was again increased, until repetitive activity was no longer able to develop (Figure 2e), the corresponding value of the depolarizing pulse was higher than the upper limit of the TRC (Figure 2f).

In Figure 2(k) the TRC is indicated first in the reference medium (R) and during the action of quinidine (R + QS) from zero to 8 min of action of quinidine. This drug decreased the TRC by a gradual decrease of the upper limit of the TRC; after 8 min of action there was no longer a value of depolarizing current which was able to elicit repetitive activity, so the TRC was zero. Another expression of the same result is shown in Figure 2(l) where the TRC obtained in Ringer solution was taken as 100% and where the percentage of this reference TRC, obtained during the action of quinidine, was plotted against the time of action of the drug. The use of a less concentrated solution of quinidine sulphate (5 μM) or of dihydroquinidine (5 μM) such as in Figure 2(m), showed the same results but here the phenomenon was

Table 3 Effect of quinidine sulphate 50 μM on the time course of repetitive activity in frog atrium

	No. of fibres	*Spike amplitude* *(mV)*	*Spike repolarization time* *(ms)*	*Spike frequency* *(Hz)*	*Spike depolarization rate* *(V/s)*	*Diastolic depolarization rate* *(V/s)*	*Difference between potential at rest and maximum diastolic potential* *mV)*
Control	11	96 ±3	749 ±10	0.85 ±0.04	1.19 ±0.07	0.050 ±0.004	15.2 ±0.4
Quinidine sulphate (50 μM)	11	67 ±2	1036 ±23	0.62 ±0.04	0.60 ±0.05	0.033 ±0.003	18.1 ±0.5
Difference from control		−29	+287	−0.23	−0.59	−0.017	+2.9
Statistical significance of differences from control (*P* value)		<0.001	<0.001	<0.001	<0.001	<0.005	<0.001

partially reversible. For these two quinidine compounds reversibility was slower than the onset of the effect.

The result obtained with quinidine was not unique. For instance procainamide (100 μM) also induced a decrease and an annulment of the TRC (Figure 2n). In contrast, when using a cardiotonic drug such as adrenaline the TRC remained nearly unchanged or was increased. On preparations showing an appreciable TRC in the reference medium, the increase of the TRC was between 0% and 20% (6 preparations) while on preparations showing a weak TRC in the reference medium, the increase was from 50 to 300% (5 preparations).

Voltage clamp conditions

The fast initial sodium current I_{Na} or fast sodium inward current

Current-voltage relationship. The result shown in Figure 3(a) was obtained by the subtraction method using three series of records (see methods section). For the determination of the reversal potential of I_{Na}, two procedures were used. The first used the current-voltage curves obtained by the subtraction method. In this case the reversal potential value was the value of potential at the intersection of the current line with the voltage axis (an important advantage of the subtraction method is that it avoids errors due to leakage current). A second determination used was based on the double pulse method. Here the inward current under study was activated. The amplitude of the stimulating pulse was chosen in such a way that it allowed the maximum amplitude of the inward current to be obtained. The duration of the stimulus was first chosen so that the inward current inactivated completely before the end of the depolarizing pulse V_1. The duration of V_1 was reduced till its end coincided with the time to peak of the inward current. In these conditions the inward current was activated till its maximum amplitude and then there was a tail current which represented the current deactivation. Then a second pulse V_2 following immediately the end of pulse V_1 was applied to the preparation and the value of depolarization of V_2 that allowed the tail current to be flat (that is the value of V_2 for which the tail current was neither inward nor outward) was the value of the reversal potential for the studied inward current activated by V_1. These two methods gave similar results. In Figure 3(a) the peak amplitude of the sodium current taken as reference b, and of the current modified by quinidine a, have been plotted as a function of the voltage to which the preparation was clamped. The curve b was obtained after about 15 to 20 min of quinidine action. The values of I_{Na} on lines a and b are the mean values observed from 12 experiments, the vertical bars represent one standard error of the mean.

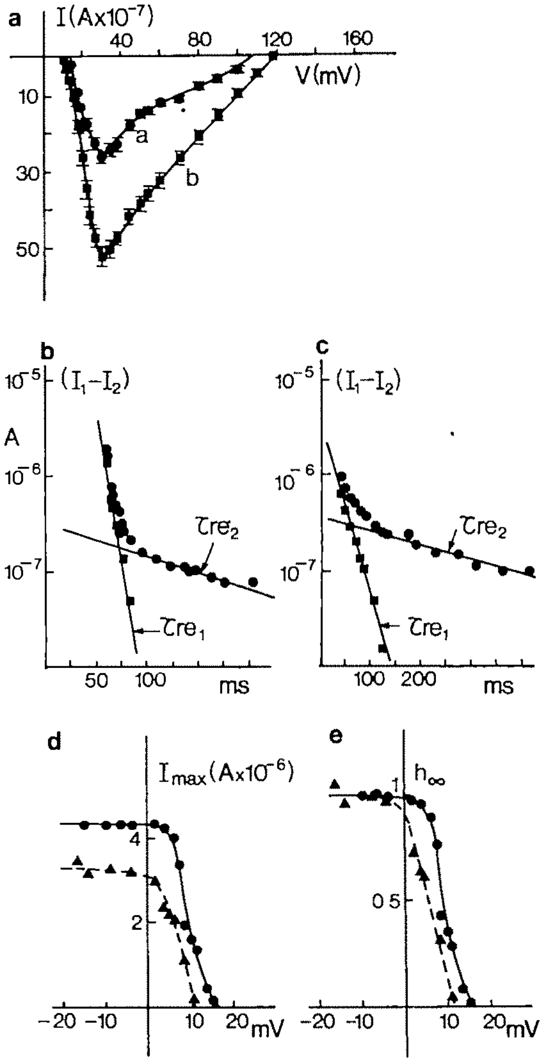

Figure 3 Modification of the fast sodium inward current by quinidine sulphate 50 μM. (a) Current-voltage relation of the sodium inward current; curve b is the reference sodium current, curve a is the sodium current obtained after quinidine action; curves a and b pass through points which are the mean amplitude values of sodium current obtained from 12 experiments. Vertical bars are one standard error of the mean. (b and c) The reactivation kinetics of the sodium inward current: (b) results obtained in a Ringer + manganese medium; there are two reactivation components with time constants τ_{re1} for the fast component and τ_{re2} for the slow component; (c) results obtained after the action of quinidine, the reactivation kinetics are decreased. (d and e) Inactivation curves of the sodium inward current: (d) I_{max} is the maximum of I_{Na} obtained either in the reference medium (continuous line) or after quinidine action (dotted line) plotted as a function of the potential to which preparation was clamped; (e) values of the steady state of the inactivation variable either in the reference medium (continuous line) or during quinidine's action (dotted line).

Quinidine decreased both the maximum amplitude of I_{Na} and the actual amplitude in response to any given clamp potential. For a depolarizing potential of 30 mV the decrease of the amplitude of I_{Na} was about 50%. The potential at which the maximum amplitude of I_{Na} was observed was not significantly altered (= +30 mV). All the experiments showed a shift of the reversal potential in the hyperpolarizing direction. The reversal potential determined in the reference medium was +118 ±1 mV and after the action of quinidine it became +109 ±1 mV ($P<0.001$, 12 preparations) (Figure 3a). The activation threshold was not in general modified or it underwent a maximum increase of only about 2 mV.

The reactivation kinetics of I_{Na}. The current studied was that obtained in a Ringer + $MnCl_2$ (2 mM) solution. The experimental procedure was based on the double step technique of Hodgkin & Huxley (1952b). Provided two pulses were sufficiently separated in time, two depolarizing pulses identical in amplitude and duration resulted in two currents of maximum amplitude. At the beginning of the experimental procedure the two pulses were applied close together. Then the second depolarization was progressively delayed until two identical currents of maximum amplitude were observed. The interval between the pulses (Δt) then measured the time for complete reactivation, I_1 being the amplitude of the current elicited by the first pulse and I_2 the amplitude of the current elicited by the second pulse.

The graph $(I_1-I_2)=f(t)$ on semi-logarithmic coordinates allowed the determination of the reactivation time constants $\tau_{re1}=6.5$ ms and $\tau_{re2}=125$ ms in the case of the experiment shown in Figure 3(b). Quinidine clearly increased τ_{re1} and τ_{re2} which became 23 ms and 295 ms respectively (Figure 3c). The results of several experiments are summarized in Table 4.

The sodium conductance g_{Na}. The general procedure has been described in the methods section. The first experiments were done by the subtraction method needing four series of records. The action of quinidine was always studied after 15 to 20 min of action. The duration of the stimulating pulses eliciting the sodium current was sufficient in each experiment to allow in every case a complete inactivation of the current before the end of the pulse. The records made in the first solution (see methods section) had to be done quickly because of an incomplete restoration of the sodium current after inhibition by TTX if this drug had acted for too long. Most of the experiments were carried out with a series of three solutions (see methods section) which were (1) Ringer + $MnCl_2$; (2) Ringer + $MnCl_2$ + quinidine; (3) Ringer + $MnCl_2$ + TTX. In both methods the preparation was stimulated by means of rectangular depolarizing pulses the amplitude of which was increased by steps of 2 mV for the low values of depolarization and then by steps of 4 mV and then of 10 mV towards a value of about 10 mV lower than the reversal potential of I_{Na}. During the experiment the reversal potential was determined by the double pulse method. After the experiment the reversal potential was checked on the current-voltage curve. The sodium conductance was calculated according to the well known following formula (Hodgkin & Huxley, 1952a):

$$I_{Na}=g_{Na}(V-V_{Na})$$

where V is the potential imposed on the preparation and V_{Na} the equilibrium potential for Na^+ ions (measured). Calculations were made by the method proposed by Jacquenod (see appendix). Those calculations allowed the following parameters to be obtained: g'_{Na}, m_∞, $1/\tau_h$, $1/\tau_m$, α_m and β_m. The curves shown are drawn by hand through the experimental points.

The results shown in Figure 4 are those corresponding to one experiment carried out on a single preparation and with a protocol using three series of records. These results are similar to those obtained in

Table 4 Effect of quinidine sulphate 50 μM on the reactivation time constants of I_{Na}

	No. of fibres	*Control (§)*	*Quinidine sulphate (†)*	*Difference from control*	*Statistical significance of differences from control (P)*
τ_{re1} ms	15 (§) 14(†)	7.2 ∓ 0.3	22.7 ∓ 0.9	+ 15.5	<0.001
τ_{re2} ms	14	105 ∓ 6	234 ∓ 15	+129	<0.001

(§) and (†): among all the experiments 15 of them (§) allowed the determination of the reactivation time constants in the control medium and after the action of quinidine the determination of the reactivation time constants could be made in 14 (†) of these experiments.

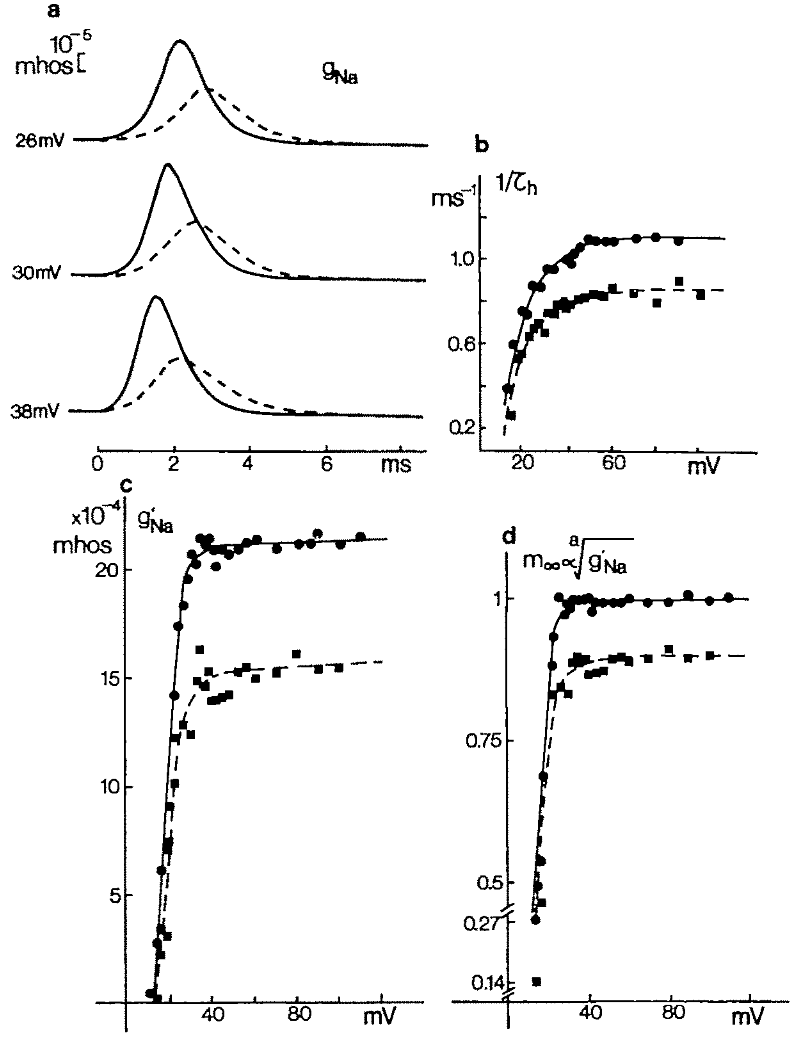

Figure 4 Modification of the conductance g_{Na} and its related parameters by quinidine sulphate 50 μM. (a) Time course of sodium conductance in a preparation clamped at +26, +30 and +38 mV. Continuous lines: g_{Na} taken as reference; dotted lines: g_{Na} modified by quinidine. Time 0 is time from which the depolarizing pulse is applied to the preparation. The continuous and interrupted lines are curves drawn through a great number of experimental points. (b) The reciprocal inactivation time constant expressed as a function of potential to which the preparation was clamped. Continuous line through (●) are reference values; dotted lines through (■) are values obtained during quinidine's action. (c) g'_{Na} is expressed as a function of the potential to which the preparation was clamped. Continuous line through (●): reference values; dotted lines through (■): decrease of g'_{Na} during quinidine's action. (d) Steady state of activation expressed as a function of the potential to which the preparation was clamped. Continuous line through (●): reference values; dotted line through (■): values obtained during the action of quinidine (on the vertical scale, value 1 corresponds to the maximum value obtained for the reference values).

4 experiments carried out with a protocol using four series of records and those obtained in 5 other experiments carried out with a protocol using three series of records. The tracings modified by quinidine were recorded after 15 to 20 min of drug action.

The time course of g_{Na} *tracings.* In Figure 4(a) the tracings of the sodium conductance plotted from a large number of experimental points are presented for three consecutive depolarizing potentials: 26 mV, 30 mV and 38 mV. The dotted lines obtained during the action of quinidine sulphate 50 μM show a decrease in the maximum amplitude of g'_{Na}, an increase of the time to maximum g_{Na}, and an increase of the time needed for its complete inactivation. These results were observed for all potentials imposed on the preparation that is from +14 to +100 mV.

g'_{Na} as a function of the imposed potential. The method for obtaining the value of g'_{Na} is described in the appendix. In the case of the results shown in Figure 4 the exponent of the activation variable m was 3 for all the imposed potentials (see appendix, equation 2). Figure 4(c) shows that quinidine produced a clear decrease of the g'_{Na} values for the whole range of imposed potentials. Therefore this graph indicates a possible effect on steady state activation (m_∞).

m_∞-potential curve. Figure 4(d) shows that steady state activation was clearly decreased in the presence of quinidine. The value of the maximum steady state (that is the plateau curve) reached by m_∞ was decreased. There was no significant shift of the curve along the x-axis.

Results concerning $1/\tau_m$, α_m and β_m. In the case of I_{Na}, for reasons given in the Introduction and in the Discussion, it was not possible to be confident that the voltage control for currents of fast kinetics was satisfactory, particularly during the beginning of the pulse. Consequently quantitative estimates for $1/\tau_m$, α_m and β_m are not given here. However, it is possible to say, on a purely comparative basis, that there was a decrease of $1/\tau_m$ for all the applied potentials in the presence of quinidine, a decrease of the values of α_m and a non-significant change of the values of β_m.

Curve $1/\tau_h$-potential. Figure 4(b) shows that quinidine induced a decrease in $1/\tau_h$ for all the potentials applied to the preparation.

Inactivation of the sodium current. The results shown in Figure 3(d) and (e) were obtained from a different preparation from that of Figure 4. The results in Figure 3(d) and (e) are similar to those obtained under the same conditions in 8 other preparations. The experimental procedure was directly derived from that used by Hodgkin & Huxley (1952b). Figure 3(e) shows that the $h_\infty=f(V)$ curve was shifted towards the hyperpolarizing potentials in the presence of quinidine. The potential allowing the complete

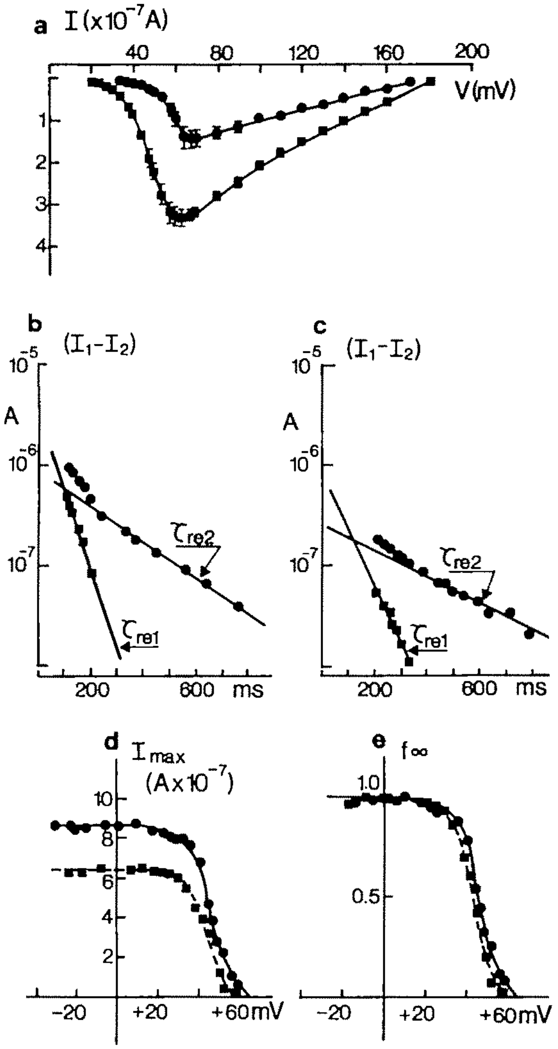

Figure 5 Modification of the slow inward calcium current and of the slow inward calcium-sodium current by quinidine sulphate 50 μM. (a) Current-voltage relationship of the calcium inward current I_{Ca}. The lower curve is the reference curve, the upper curve is the current obtained during quinidine's action. The two curves pass through points which are the mean values of the amplitude of the calcium current obtained from 10 experiments. Vertical bars show standard error of the mean. (b and c) Reactivation kinetics of the slow calcium-sodium inward current: (b) results obtained in a Ringer+TTX medium. There are two reactivation components whose time constants are τ_{re1} for the fast one and τ_{re2} for the slow one. (c) Results obtained during the action of quinidine added to a Ringer+TTX medium. There is an increase of the time constants of the two reactivation components. (d and e) The inactivation of the slow inward current: (d) I_{max} is the maximum of I_{Ca} obtained either in the reference medium (continuous line) or during quinidine's action (dotted line) plotted as a function of the potential to which the preparation was clamped. (e) Values of the steady state inactivation variable either in the reference medium (continuous line) or during the action of quinidine (dotted line).

inactivation of I_{Na} was shifted by 4 to 5 mV in the hyperpolarizing direction. A similar shift of the inactivation curve in the same direction is seen in Figure 3(d). Moreover a decrease of the steady state of I_{max} (upper part of the curve) in the presence of quinidine can be observed.

The calcium current and the calcium-sodium current

Current-voltage relation of the calcium current I_{Ca} (Figure 5a). To obtain the current-voltage relation of I_{Ca} the general protocol was similar to that used for I_{Na} but the media used were different. Results shown in Figure 5(a) were obtained by the subtraction method using three series of records (see methods section), the three media being successively (1) Ringer solution without Na^+; (2) Ringer without Na^+ + quinidine and (3) Ringer without $Na^+ + MnCl_2$. It was not possible to determine the reversal potential of I_{Ca} during the experiments, by the double pulse method. Because of the slow kinetics of this current, the outward current often began to be partially activated at the end of the first pulse V_1, so that it was impossible to obtain a flat tail of current during the second pulse V_2. Thus the reversal potential of I_{Ca} was only determined from the current-voltage curves.

The continuous curve drawn in Figure 5(a) was obtained after about 15 to 20 min of action of quinidine sulphate 50 μM. The values of I_{Ca} are the mean values observed from 10 experiments. The vertical bars represent the standard error of the mean. The maximum amplitude of I_{Ca} was decreased (upper curve), and for a 70 mV amplitude stimulus applied to the preparation the decrease was about 57%. The potential at maximum amplitude was shifted slightly positively (about 4 mV); the reversal potential was shifted in the hyperpolarizing direction (about 10 mV). The reversal potential determined in the reference medium was +181 ±2 mV, and after the action of quinidine it was +171±2 mV ($P<0.005$, 10 preparations). The potential threshold of I_{Ca} was often higher in the presence of quinidine.

Reactivation kinetics of the slow inward calcium-sodium current. The current studied was that obtained in Ringer + TTX. The experimental method was the same as that used for I_{Na}. The pulses used to elicit the slow inward current were longer than for I_{Na} and lasted long enough to allow a complete inactivation of this slow current. The experiment shown in Figure 5(b) and (c) indicates that quinidine increased the reactivation time constants. Thus in the reference medium the reactivation time constants were $\tau_{re1} = 59$ ms and $\tau_{re2} = 256$ ms; after about 20 min of quinidine action these values became respectively 80 and 345 milliseconds. From a summary of all experiments of this type it was possible to establish that quinidine decreased the reactivation kinetics of the slow inward current (see Table 5).

The calcium conductance g_{Ca}. Here, it should be recalled that the results of Besseau (1972) obtained on frog auricular preparations showed that the Hodgkin-Huxley model can be successfully applied to the calcium conductance, the g_{Ca} time courses calculated from the Hodgkin and Huxley parameters allowing a good fit to the experimental points.

As for I_{Na} two experimental procedures were tried in order to obtain a relatively 'pure' I_{Ca} and to calculate the calcium conductance. The first experiments were carried out with a subtraction method using four series of records after testing the state of the preparation (see methods section). A series of records was made in each medium. The amplitude of the depolarizing pulses was increased by steps of 4 mV and then of 10 mV in order to have enough points to draw the m_∞-potential curve. Particularly because of the time needed to do such experiments and because of the difficulty of recovery of I_{Ca} after the first solution, a second type of experiment with a subtraction method using three series of records was preferred (same solutions used as for the inward calcium current). An example of the results obtained with such a method is shown in Figure 6(a–d). These

Table 5 Effect of quinidine sulphate 50 μM on the reactivation time constants of the calcium-sodium inward current of slow kinetics

	No. of fibres	*Control*	*Quinidine sulphate*	*Difference from control*	*Statistical significance of difference from control (P)*
τ_{re1} ms	14	94 ∓ 7	127 ∓ 9	+ 33	<0.01
τ_{re2} ms	14	305 ∓ 11	412 ∓ 15	+107	<0.001

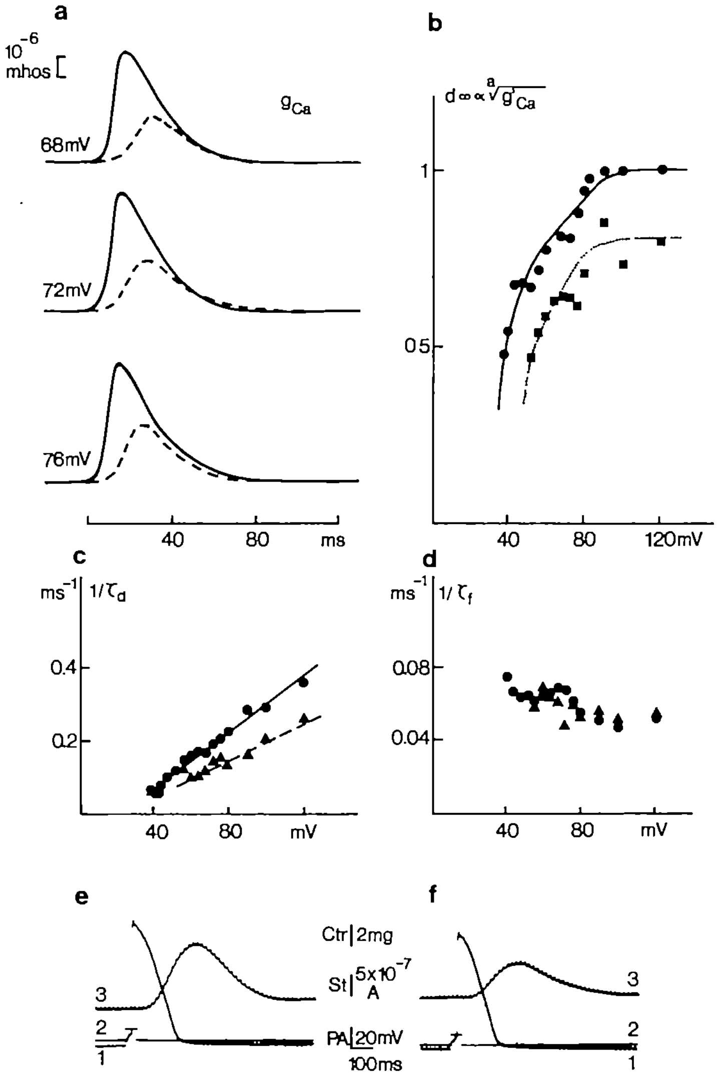

Figure 6 Modification of the calcium conductance and of its related parameters by the action of quinidine sulphate 50 μM. (a) Calcium conductance time course for 68, 72 and 76 mV depolarizations imposed on the preparation. Continuous line: g_{Ca} reference time course; dotted line: g_{Ca} time course obtained after action of quinidine. The continuous and interrupted lines are curves drawn through a great number of experimental points. (b) Steady state of activation expressed as a function of the potential to which the preparation was clamped. Continuous line through filled circles: values of d_∞ taken as reference; dotted line through filled squares: values obtained during quinidine's action (on vertical scale 1 corresponds to the maximum value of d_∞ obtained in the reference medium). (c) Values of $1/\tau_d$ as a function of the potential to which the preparation was clamped. Continuous line through (●): reference values; dotted line through (▲): values obtained during quinidine's action. (d) Values of $1/\tau_f$ as a function of the potential to which the preparation was clamped: (●): reference values; (▲): values obtained during the action of quinidine. (e and f) The action of quinidine on the mechanical activity of frog cardiac auricular trabeculae: (e) simultaneous recordings of the action potential (trace 1) elicited by a depolarizing current (trace 2) and of the contraction (trace 3) recorded with a RCA 5734 transducer in the reference medium: (f) the same recordings made after 25 min of action of quinidine. Notice the decrease in the peak amplitude (PA) of contraction.

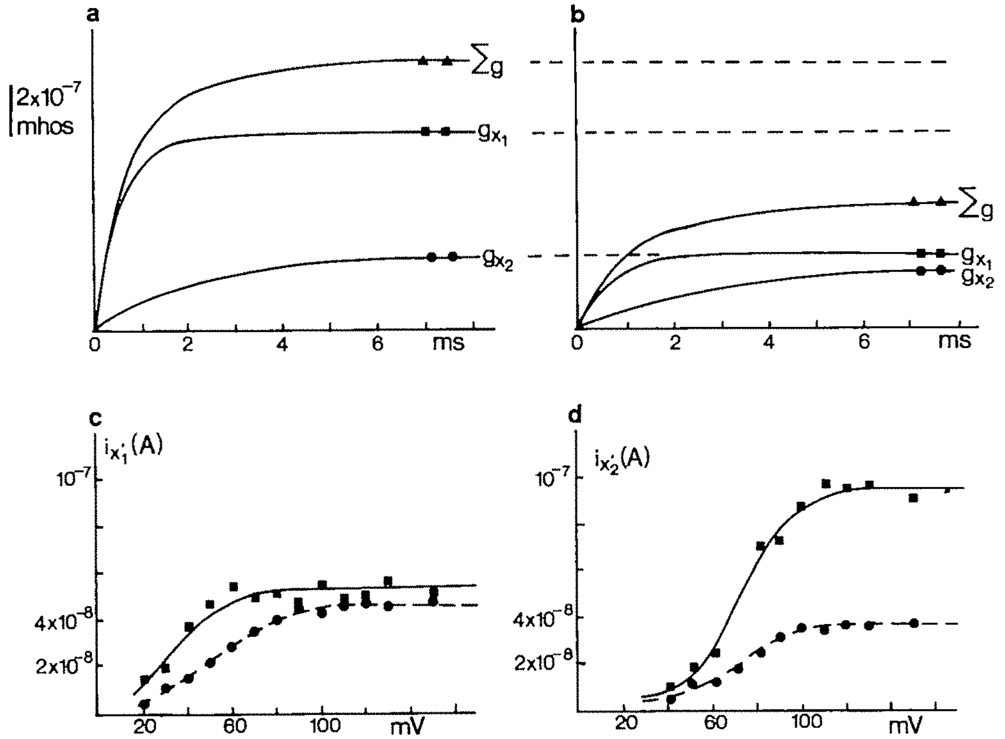

Figure 7 The delayed conductances and their activation curves. (a and b) The delayed conductances time courses: (a) in reference medium, g_{x1} is the fast component labelled with two filled squares; g_{x2} is the slow component labelled with two filled circles: Σg is the sum of g_{x1} and g_{x2} labelled with two triangles; (b) g_{x1}, g_{x2} and Σg time courses after quinidine's action. Notice the strong decrease of the amplitude of g_{x1}. (c and d) The activation curves of the fast component (c) and of the slow component (d) of the total delayed outward current. Continuous lines through (■): reference values; dotted lines through (●): values obtained during quinidine's action.

results are similar to those obtained in 3 experiments carried out with a protocol using four series of records and those obtained in 5 other experiments carried out with a protocol using three series of records. The calculations of the parameters concerning g_{Ca} were similar to those carried out for g_{Na}. The determination of the exponent 'a' of the activation variable d led to higher values than for g_{Na}; the values encountered for 'a' ranged from 6 to 14.

g_{Ca} *time course.* Figure 6(a) shows that quinidine sulphate 50 µM decreased the maximum amplitude of g_{Ca}, increased the time to reach maximum, while it did not seem to modify significantly the time needed for the complete inactivation of g_{Ca}.

d_∞*-potential curve.* In Figure 6(b) a clear decrease of the steady state of the activation in the presence of quinidine is shown. This result applies to all potentials applied to the preparation. The threshold of d_∞ was higher, the shift being about 8 to 10 mV towards more positive potentials.

$1/\tau_d$*-potential curve.* Quinidine induced a decrease of the values of $1/\tau_d$, that is, an increase of the activation time constant (Figure 6c).

The rate constants α_d *and* β_d. A clear decrease of the values of α_d was observed in the presence of quinidine; the greater the imposed potential, the greater the decrease. There was also a decrease of the values of β_d, this decrease being less than for α_d and not always significant.

$1/\tau_f$*-potential curve.* Quinidine did not significantly alter the values of $1/\tau_f$ (Figure 6d).

Inactivation of the inward calcium-sodium current of slow kinetics. The current was studied in a

Ringer + TTX medium. In frog auricular trabeculae, the calcium component seems to be greater than the sodium component (Besseau, 1971), both constituting the slow current. The results shown in Figure 5(d) and (e) were obtained from a different preparation from that of Figure 6(a–d). The results reported in Figure 5(d) and (e) are similar to those obtained under the same conditions in 7 other preparations. Figure 5(d) shows that quinidine induced a decrease of the steady state of I_{max} (plateau of the curve). In addition there was a slight shift of the curve towards hyperpolarizing potentials. Figure 5(e) indicates that quinidine did not modify the inactivation curve, there being only a slight and insignificant shift of the curve towards hyperpolarizing potentials.

The delayed system ($i_{x1} + i_{x2}$) and the corresponding conductances ($g_{x1} + g_{x2}$). The analysis of the total delayed current recorded on the auricular trabeculae of frog showed the existence of two components i_{x1} and i_{x2}, the first having fast activation kinetics and the second slow activation kinetics (Brown & Noble, 1969a, b). The reversal potential of the first component seemed to vary between +5 (or less) and +15 mV with regards to the holding potential taken as zero. The reversal potential of the second component was often between +30 and +40 mV or more. In some preparations only one component of the delayed outward current was found. The effect of quinidine was only studied on preparations showing two components of this current. To obtain the results, the method indicated in the appendix is directly derived from that of Brown & Noble (1969a, b) and Noble & Tsien (1969a, b).

The current traces obtained in a Ringer + TTX + $MnCl_2$ medium appeared to be the sum of two exponential functions. The measurement of the two reversal potentials V_{x1} and V_{x2} of the two components gave the value of the two conductances $g_{x1} = i_{x1}/(V - V_{x1})$ and $g_{x2} = i_{x2}/(V - V_{x2})$. The activation time constants of the fast and slow systems were obtained from the following relations: $\alpha_{n1} = n_\infty 1/\tau_{n1}$; $\alpha_{n2} = n_\infty 2/\tau_{n2}$; $\beta_{n1} = (1 - n_{\infty 1})/\tau_{n1}$; $\beta_{n2} = (1 - n_{\infty 2})/\tau_{n2}$. The values of g_{x1} and g_{x2} determined at time zero on semi-logarithmic plots express the steady state activation of each conductance component. The activation curves of the delayed systems were obtained from the two components of the tail currents when these two components were plotted on semi-logarithmic scales. Then it was possible to determine the ordinates at time zero: i'_{x1} for the fast component i_{x1}, and i'_{x2} for the slow component i_{x2}. These ordinates at the origin would be representative of the initial state of the deactivation state n_∞ (at the steady state) of the current components obtained during the preceding stimulus (Noble & Tsien, 1969a).

The results shown in Figures 7(a) and (b) and 8(a–d) were obtained in the same preparation and are representative of those obtained in 8 other preparations. The results indicated in Figure 7(c) and (d) were obtained in different preparations and are similar to those obtained from 7 other preparations.

The conductances g_{x1} and g_{x2}. Quinidine induced an important decrease of the amplitude of the g_{x1} component and a weak decrease of the amplitude of the g_{x2} component, leading to an important decrease of their algebraic sum Σg. This effect was the same for all the potentials applied to the preparation (Figure 7a and b).

The activation time constants of g_{x1} and g_{x2} components. Quinidine decreased $1/\tau_{n1}$ particularly for the potential values higher than +70 mV. The values of $1/\tau_{n2}$ were slightly decreased over the whole range of potentials applied (Figure 8a and b).

The activation rate constants of the g_{x1} and g_{x2} components. Quinidine clearly decreased the values of α_{n1} and α_{n2} but did not significantly alter the values of β_{n1} and β_{n2} (Figure 8c and d).

The activation curves of the i_{x1} and i_{x2} components of the delayed outward current. Quinidine decreased the values of i'_{x1}. The half activation potential was increased about 15 mV. There was a very clear decrease of the values of i'_{x2} while the half activation potential was not significantly modified (Figure 7c and d).

The reversal potential of i_{x1} and i_{x2}. From 14 experiments it can be said that in the reference medium the reversal potential of i_{x1} component was $+13 \pm 1$ mV and that of the i_{x2} component $+39 \pm 1$ mV. Quinidine decreased slightly the value of i_{x1} reversal potential which became $+10 \pm 1$ mV ($P < 0.05$) and the value of i_{x2} reversal potential was decreased to $+34 \pm 1$ mV ($P < 0.005$).

Discussion

Two types of results have been obtained: those obtained in current clamp conditions and in voltage clamp conditions. Results obtained in voltage clamp conditions allowed a fairly good understanding of those obtained in current clamp conditions and of results obtained with microelectrode recordings by other authors.

Serious strictures have been made concerning the validity of voltage clamping by the double sucrose gap technique (Johnson & Lieberman, 1971). In addition to errors recalled by Besseau (1972) the presence of an external series resistance (Rougier *et al.*, 1968) must be mentioned, the notable role of which has been

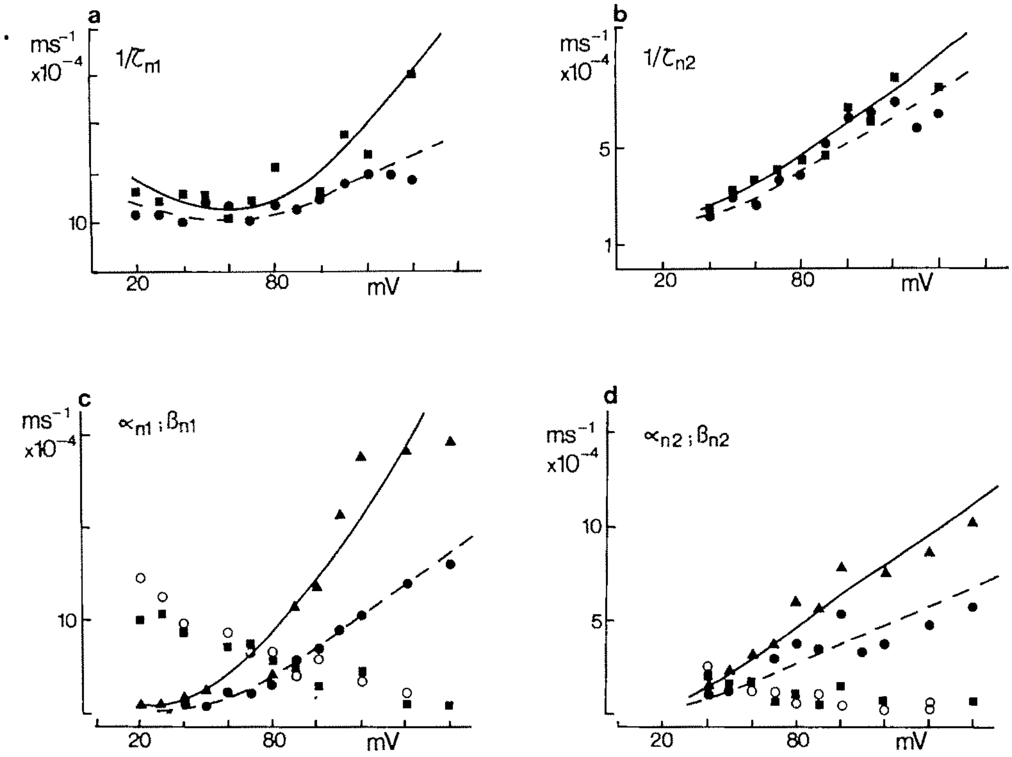

Figure 8 The activation parameters of the delayed conductances g_{x1} and g_{x2}. (a and b) The reciprocal activation time constant of the delayed conductance component: $1/\tau_{n1}$ for the fast component in (a) and $1/\tau_{n2}$ for the slow component in (b). Continuous line through (■): reference values; dotted line through (●): values obtained during action of quinidine sulphate 50 μM. (c and d) The activation rate constants of the fast delayed conductance α_{n1} and β_{n1} (c) and of the slow delayed conductance α_{n2} and β_{n2} (d). Symbols: (▲) and continuous line are values of α_{n1} or α_{n2} in the reference medium; (●) are values of α_{n1} or α_{n2} after quinidine's action; (○) are the values of β_{n1} or β_{n2} in the reference medium and (■) are values of β_{n1} or β_{n2} during quinidine's action.

emphasized by Léoty & Poindessault (1974) and by Poindessault, Duval & Léoty (1975). In view of this it must be recognized that the control of the potential of the membrane in the central compartment of the preparation is suspect. It is necessary to point out that I have not made a quantitative analysis but have tried to identify the components of the ionic conductances which might be affected by the action of quinidine. The results must be discussed from a strictly qualitative viewpoint and the major interest of the results comes from the comparisons which could be made between reference results and those obtained during the action of quinidine.

The above considerations and criticisms apply particularly to experiments performed with double sucrose gap devices with a large central compartment and without vaseline seals. Recent work by de Hemptinne (unpublished observations) leads to the conclusion that a double sucrose gap technique using partitions of vaseline is better as regards the errors made with a double sucrose gap technique with no physical partitions between solutions (this remark being valid because of the same test compartment width used, as in the present work, that is 150 μm, but with the restriction that de Hemptinne used an electronic circuitry allowing a compensating procedure of the series resistance in the central compartment).

Before discussing the results it should be recalled that in the frog auricular preparation a voltage and time-dependent inward current of fast kinetics carried by sodium ions is mainly responsible for the depolarizing phase of the AP (Rougier *et al.*, 1969). A time and voltage-dependent inward current of slow kinetics carried both by sodium and calcium ions is mainly responsible for the final depolarization phase and the plateau phase of the AP (Rougier *et al.*, 1969). The calcium component is of greater amplitude than the sodium component (Besseau, 1971). The repolarization phase of the AP can be explained by the existence of a delayed rectification (Noble, 1962, 1966; McAllister & Noble, 1966, 1967; Rougier *et al.*, 1968); potassium ions would constitute a part of this current which has very slow kinetics (Besseau, 1972).

The decrease of the amplitude of I_{Na} as of g_{Na} in the presence of quinidine simply explains the decrease of the amplitude of the AP. The reactivation time constants of I_{Na} were increased by quinidine; thus this drug lengthened the time required for the membrane to return to its fully activated state. This delay of reactivation explains the lengthening of the effective refractory period observed with quinidine by Vaughan Williams (1958) in the absence of any prolongation of the AP duration. The decrease of m_∞ expresses a reduced total capacity of the cardiac membrane to carry sodium ions. The increase of τ_m, expressing an increased delay of the g_{Na} activation, accounts for the decrease of the rate of rise of the AP and would result in a slower spread of intracellular current responsible for propagation of impulses through the myocardium. The reduced values of α_m in presence of quinidine help to understand the slowing down of the activation process. The increase of τ_h, expressing a delay in the inactivation of g_{Na}, accounts for the increase observed in the total time needed for the inactivation of g_{Na}. This result partly explains the increase of reactivation time constants of I_{Na}. The shift of the h_∞-potential curve in the hyperpolarizing direction would cause a decrease of the fraction of the sodium carrying system in non-inactivated state. Quinidine would thus maintain a partial inactivation of this system.

The decrease of the amplitude of the slow inward calcium current can explain the negative inotropic effect of quinidine observed in Figure 6(e) and (f), because according to Léoty, Raymond & Gargouïl (1970) the mechanical response of the frog auricular fibre depends, for its 'phasic' component, on the entry of calcium from the external medium. The decrease of the amplitude of the contraction is in agreement with the depressant effect on myocardial contractility recalled by Thorpe (1973) and established by Vaughan Williams (1958), Angelakos & Hastings (1960) and Parmley & Braunwald (1967). The delayed reactivation of the total (calcium + sodium) slow inward current could account for the failure of responses to a high stimulus frequency in the presence of quinidine, when the action potentials would normally have developed a high level of activation of calcium-sodium current. Therefore quinidine increases the time needed for a total reavailability of the membrane for the transport of the fast and slow inward currents. This effect would both contribute to an increase of the relative refractory period, and would oppose the maintenance of responses to an ectopic pacemaker.

The decrease of the maximum amplitude of g_{Ca} can partially explain the depression of the plateau of the AP and of the spike amplitude during repetitive activity, both observed in presence of quinidine. The decrease of d_∞ would express, in Hodgkin–Huxley terms, a decrease of the proportion of the activating molecules for calcium transport in the *a* state. Although the increase of the activation time constant of g_{Ca} could not account for the slowing of MRD, it could nevertheless explain the gradual 'rounding' of the final phase of depolarization of the AP, i.e. in Hodgkin–Huxley terms, α_d is decreased.

The decrease of the amplitude of g_{x1} partially explains the lengthening of the final phase of the AP observed in the presence of quinidine. The increase of τ_{n1} could contribute to the increase of the maximum repolarization time of the spikes during repetitive activity observed in presence of quinidine. The effects of this drug on g_{x2} amplitude however were small; moreover in view of the much slower activation kinetics of g_{x2}, it is doubtful whether a modification of this system could contribute to the action of quinidine on the time course of the AP. However, the increase in the half-activation potential for i_{x1} indicates that higher depolarizations would be required to activate the latter system, and this delay of i_{x1} activation may be considered partly responsible for the delayed repolarization.

The results obtained in the present work are clearly useful for a better understanding of the modifications induced by quinidine on both the AP and the repetitive activity. Concerning the AP the present results are in general agreement with the finding of a decrease of the rate of rise produced by quinidine observed by Weidmann (1955) on Purkinje fibres of sheep and calf; they also confirm those obtained by Vaughan Williams (1973) using intracellular microelectrodes impaled in rabbit auricular muscle. Since the rate of rise of the AP affects the conduction velocity (Trautwein, 1963), the decrease of the rate of rise of the AP by quinidine partially explains the slowing of conduction observed with quinidine (Vaughan Williams, 1958; Szekeres & Papp, 1971). Quinidine induced a lengthening of the final repolarization phase of the AP. The maximum value of the plasma concentration of quinidine observed clinically is near 10 μM, a concentration at which there is only a little change in AP duration. For example, a concentration of 9 μM quinidine induced only a small lengthening of the 'tail of the repolarization phase' of the AP (Vaughan Williams, 1958). In fact, on the frog auricular preparation, the concentration required to alter significantly the conductance parameter was 50 μM, so the lengthening of the final repolarization phase of the AP observed with 50 μM quinidine can hardly explain the lengthening of the effective refractory period but this last effect has already been explained by the increase of the reactivation time constants of both fast and slow inward currents. So it becomes possible to understand why a lengthening of the effective refractory period can be observed even when the AP duration remains unchanged (Vaughan Williams, 1973).

The repetitive activity elicited by depolarizing currents in frog auricular preparations depends on the deactivation of a delayed outward system with fast kinetics (Brown & Noble, 1969a) and on the activation of a slow inward system (Lenfant, Mironneau & Aka, 1972). The decrease of the spike amplitude during repetitive activity can be partly explained by the decrease of the values of d_∞ and therefore the decrease of the maximum amplitude of g_{Ca}. The inactivation time constant of g_{Ca} seems not to be modified, so one can assign the increase of the maximum repolarization time of the spikes to the increase of the activation time constant of the g_{x1} component of the delayed conductance. The decrease of the spike depolarization rate can be partially explained by the increase of the activation time constant τ_d of g_{Ca}. The decrease of the diastolic depolarization slope is perhaps better understood if one takes into account the decrease of the amplitude of g_{x1}. Component g_{x1} being less activated, the conductance is therefore smaller during activation but also during deactivation, so that it would result in a longer time to reach the activation threshold of the slow inward system. The increase of each spike duration and the decrease of the diastolic depolarization slope explain the decrease of the firing frequency of repetitive activity. Eventually after quinidine has acted for sufficient time and all the effects reported above are sufficiently established a total abolition of repetitive activity is to be expected. It would then be interesting to study the effects of new drugs on the triggering range of current (TRC). It could test whether a drug has or has not the ability to reduce or depress repetitive activity, experimentally triggered by a depolarizing current. This procedure might prove useful as a screening test for drugs able to suppress ectopic pacemakers or extrasystoles.

In spite of the limitations of the method used in the present work, reproducible variations of the apparent values of the parameters have been observed on frog auricular preparations in the presence of quinidine. Such results may be useful in elucidating the mode of action of drugs. It is noteworthy that results obtained in voltage clamp conditions aid clearly in the interpretation of results obtained in current clamp conditions. It should also be pointed out that the conclusions drawn from these experiments with the double sucrose gap technique are in excellent agreement with results obtained previously with intracellular microelectrodes. It seems reasonable therefore to suggest that similar or related types of study may be of use in the attempt to understand better the mode of action of cardioactive drugs, and particularly of antiarrhythmic agents, on the electrical properties of cardiac membranes.

This work was performed in the C.N.R.S. associated team No. 111. I am grateful to Professor Y.M. Gargouïl for suggesting this work and continuously following and criticizing it. I should like to thank Dr E.M. Vaughan Williams for his help in writing this paper and particularly for his very constructive criticisms. I thank also Dr J.C. Jacquenod for his help with the calculations and Dr J.P. Poindessault for demonstrating the double sucrose gap technique, both essential for this work. I am grateful to Mrs P. Régondaud and Miss M. Moreau for their skilled technical assistance.

Appendix

Sodium (as calcium) conductance

The sodium current was obtained by subtraction of a tracing obtained in a medium allowing the development of I_{Na} from a tracing obtained in a medium where I_{Na} was inhibited (Figure 9). The calculations made, assumed that the conductances can be described reasonably well by the Hodgkin and Huxley equations proposed for the giant axon of the squid (Besseau, 1972). The method that has been used and the corresponding programmes have been elaborated by J.C. Jacquenod (Assistant, Laboratoire de Physiologie Animale, Faculté des Sciences de l'Université de Poitiers, France: method and results to be published).

It was possible to calculate the conductance g_{Na} from the reversal potential of I_{Na} measured during the experiment and the imposed potential. In Figure 9(b), the sodium conductance obtained for a depolarizing pulse of 40 mV is drawn as a function of time (the beginning of the pulse being time zero). The relation between the current and the corresponding conductance given by Hodgkin & Huxley (1952a) is:

$$g_{Na} = I_{Na}/(V - V_{Na}) \qquad (1)$$

where I_{Na} is the amplitude of the current, V the imposed potential and V_{Na} the equilibrium potential for Na ions. Hodgkin & Huxley (1952c) made the assumption that g_{Na} was a function depending on two variables: m, the activation variable and h, the inactivation variable:

$$g_{Na} = m^a \, . \, h^i \, . \, \overline{g_{Na}} \qquad (2)$$

where $\overline{g_{Na}}$ is a constant and is the absolute maximum value that can be reached by g_{Na}. The exponent i of h is assumed to be 1. m and h variables yield to the following differential equations:

$$dm/dt = \alpha_m(1-m) - \beta_m \, . \, m \qquad (3)$$

$$dh/dt = \alpha_h(1-h) - \beta_h \, . \, h \qquad (4)$$

where m represents the proportion of activating molecules in the α state, (1-m) the proportion of activating molecules in the β state, h the proportion of inactivating molecules in the α state and (1-h) the proportion of inactivating molecules in the β state. m and h are dimensionless variables which can vary between 0 and 1 (Hodgkin & Huxley, 1952c). The solutions of equations (3) and (4) which satisfy the

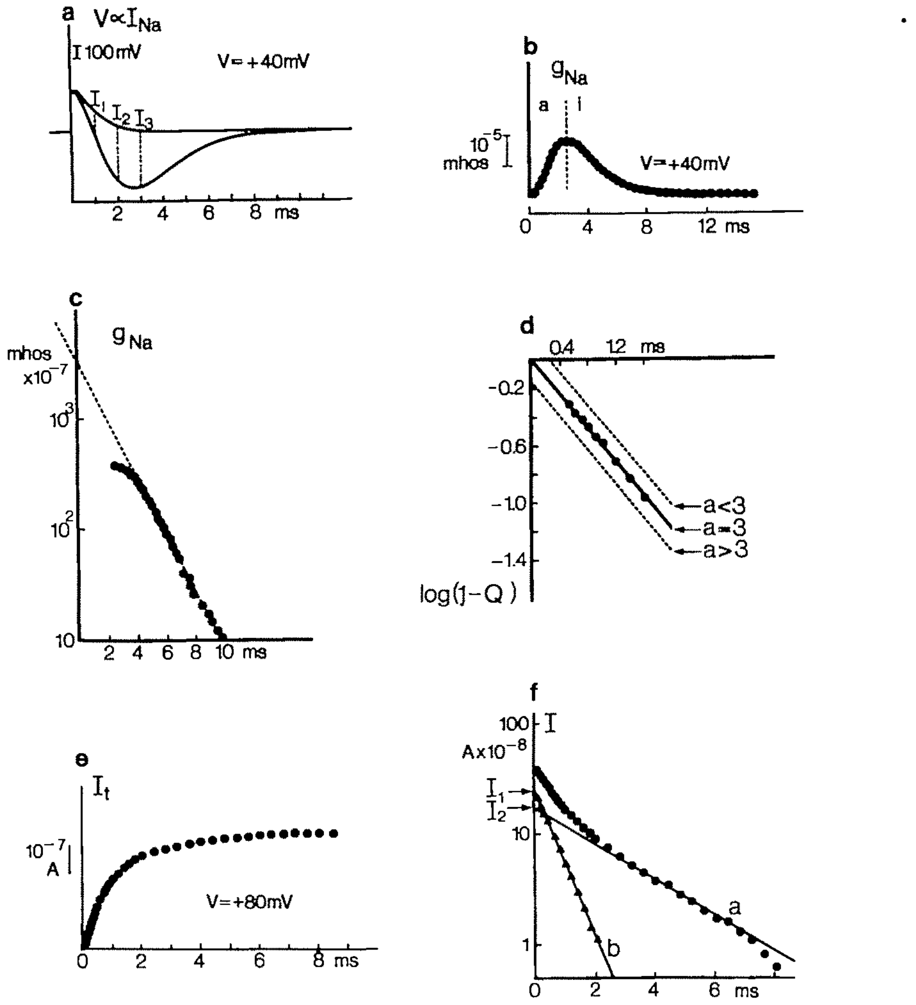

Figure 9 Calculation method used for ionic conductances and related parameters. (a) To obtain the sodium current values two current tracings obtained for a 40 mV depolarizing pulse were superimposed. The horizontal line on the left of the y-axis is the zero value of the current. Time zero corresponds to the beginning of the depolarizing pulse. Values I_1, I_2, $I_3 \ldots I_n \ldots$ are obtained from the subtraction of the two superimposed current curves (upper curve being obtained when the current is inhibited). (b) g_{Na} time course. The values calculated for g_{Na} are plotted against time; time zero is the beginning of the stimulating pulse; a='activation phase', i='inactivation phase'. (c) Determination of g'_{Na} and $1/\tau_h$. As explained in the text the values of g_{Na} during the inactivation phase (b) are plotted in (c) on a semi-logarithmic scale. A linear regression on the aligned points enables the values of $-1/\tau_h$ to be obtained. The value of the ordinate at the origin gives g'_{Na}. (d) Determination of exponent 'a' of the activation variable m. Values of 'a' higher than 3 and values of 'a' below 3 lead to lines which do not pass through the origin. In (d) only a=3 allows a straight line to pass through the origin; therefore the value a=3 was taken for the following calculations concerning this experiment. The results shown in (d) come from those of (a), (b) and (c). (e) The values of the total delayed outward current I_t obtained for a depolarizing pulse of +80 mV are plotted against time. Time zero corresponds to the beginning of the stimulating pulse. (f) The values of current (I) obtained by subtraction of each of the experiment values from the maximum value of I_t (e) are plotted against time. Two linear regressions allow the determination of I_1 and I_2 and the slopes $-1/\tau_{n1}$ and $-1/\tau_{n2}$ of the two individualized components.

following conditions $m=m_0$ and $h=h_0$ at $t=0$, are:

$$m = m_\infty - (m_\infty - m_0)\exp(-t/\tau_m) \qquad (5)$$

where $m_\infty = \alpha_m/(\alpha_m + \beta_m)$ and $\tau_m = 1/(\alpha_m + \beta_m)$ = activation time constant and

$$h = h_\infty - (h_\infty - h_0)\exp(-t/\tau_h) \qquad (6)$$

where $h_\infty = \alpha_h/(\alpha_h + \beta_h)$ and $\tau_h = 1/(\alpha_h + \beta_h)$ = inactivation time constant.

At rest, that is at time $t=0$ and before ($t=0$ is the beginning of the depolarizing pulse), g_{Na} is very small and it can be considered negligible if it is compared with the g_{Na} value reached during a large depolarization (so $m_0=0$). Then we have:

$$m = m_\infty - m_\infty \exp(-t/\tau_m) = m_\infty(1 - \exp(-t/\tau_m))$$

At time $t=\infty$, inactivation can be considered nearly complete and g_{Na} is negligible, so h_∞ is taken as zero; thus, $h = h_0 . \exp(-t/\tau_h)$ and equation (2) becomes:

$$g_{Na} = \overline{g_{Na}} . [m_\infty(1 - \exp(-t/\tau_m))]^a . \\ . [h_0 . \exp(-t/\tau_h)]^i \qquad (7)$$

Taking

$$g'_{Na} = \overline{g_{Na}} . m_\infty^a . h_0^i \qquad (8)$$

the relation (7) therefore becomes:

$$g_{Na} = g'_{Na}(1 - \exp(-t/\tau_m))^a . \exp(-t/\tau_h) \qquad (9)$$

g'_{Na} represents the value that g_{Na} would be able to reach if inactivation would be maintained at its resting level h_0 (in absence of inactivation). After the maximum value of g_{Na}, $\exp(-t/\tau_m)$ tends towards 0 and that carries $1-\exp(-t/\tau_m)$ near 1, so g_{Na} becomes:

$$g_{Na} = g'_{Na} . \exp(-t/\tau_h) \qquad (10)$$

With equation (10) it became easy to determine the values of τ_h and g'_{Na} by plotting the values of g_{Na} during the inactivation phase of g_{Na} on semi-logarithmic paper (Figure 9c). The slope of the straight line determined by means of a linear regression is $-1/\tau_h$ and the value of the ordinate at the origin gives g'_{Na}. By doing such calculations for all the depolarization values applied to the preparation during the experiment it was possible to draw the curves $g'_{Na}=f(V)$ and $1/\tau_h = f(V)$. Since g'_{Na} is known it became possible to obtain a number proportional to m_∞ from equation (8) where $\overline{g_{Na}}$ is a constant. During all the experiments h_0 was a constant because between depolarizing pulses the resting potential was constant. Taking $\overline{g_{Na}} . h_0 = K$, then $m_\infty^a = g'_{Na}/K$, $m_\infty \alpha \sqrt[a]{g'_{Na}}$. Therefore, it was possible to draw the curve $m_\infty = f(V)$. 'a' and 'i' are the exponents of the activation and inactivation variables. The experiments showed that 'i' had the value 1. It was necessary to determine the values of 'a' which could account for the activation function. From equation (9) can be obtained:

$$-t/\tau_m = \log[1 - (g_{Na}/g'_{Na} . \exp(t/\tau_h))^{1/a}]$$

Taking $Q = (g_{Na}/g'_{Na} . \exp(t/\tau_h))^{a-1}$ the relation becomes:

$$(-1/\tau_m) . t = \log(1-Q) \qquad (11)$$

which is an equation of a straight line passing through the origin,

where $$\tau_m = -t/\log(1-Q) \qquad (12)$$

So if 'a' is accurately determined the curve $\log(1-Q)=f(t)$ is a straight line crossing the ordinate at the origin as can be seen for 'a' = 3 in the case of the experimental points shown in Figure 9d.

More, equation (12) allows the calculation of τ_m and then the curve $1/\tau_m = f(V)$ can be constructed. If the values of α_m and β_m are needed they can be calculated from $\alpha_m = m_\infty/\tau_m$ and $\beta_m = (1-m_\infty)/\tau_m$.

Outward current and delayed conductance

The outward current was assumed to be the sum of two exponential functions of time as did Noble & Tsien (1969a) for their i_{x1} and i_{x2} currents elicited in the plateau range of potentials in cardiac Purkinje fibres. The currents recorded here were identical with the first and second components of current studied by Brown & Noble (1969a) in frog atrial muscle. The equations used were derived from those used by Hodgkin & Huxley (1952c) for their study of g_K on nerve. The total outward current was recorded for various values of depolarizing pulses. An example of total outward current obtained with a +80 mV pulse is drawn against time in Figure 9(e) (similarly the tail currents obtained at the end of a stimulating pulse were drawn). For each value of time, subtraction of the experimental values of current from the maximum value of I_t (the value at the end of the pulse) gives the values of current I which were plotted on a semi-logarithmic scale as in Figure 9(f). A straight line (a) passing through experimental points and determined by a linear regression, allowed to determine the time constant τ_{n2} of the delayed system of slow kinetics and to determine the ordinate at the origin I_2. Then the values of the points situated on the straight line (a) were subtracted from the corresponding experimental values. This led to another series of points and another linear regression was used to determine the position of a second straight line (b) crossing the y-axis at the value I_1. From straight line (b) it was possible to determine the time constant τ_{n1} of the delayed system of fast kinetics. With the reversal potential of the i_{x1} and i_{x2} components measured during the experiment it was easy to calculate the g_{x1} and g_{x2} conductance values from the following equations:

$$g_{x1} = i_{x1}/(V - V_{x1}) \quad \text{and} \quad g_{x2} = i_{x2}/(V - V_{x2}) \qquad (13)$$

where V_{x1} and V_{x2} are the reversal potentials of i_{x1} and i_{x2}, and V the imposed potential. The determination of the activation time constants τ_{n1} and τ_{n2} of the two conductances allowed the curves $1/\tau_{n1}=f(V)$ and $1/\tau_{n2}=f(V)$ to be drawn.

Now considering one of the two delayed systems g_{x1} or g_{x2} (that is in general g_x) we made the assumption that:

$$g_x = \overline{g_x} \cdot n^b \qquad (14)$$

and

$$dn/dt = \alpha_n(1-n) - \beta_n \cdot n \qquad (15)$$

where $\overline{g_x}$ is a constant, b has the value 1 (a value of one fits the experimental points well), n is the activation variable and it varies between 0 and 1. n would represent the proportion of particles (molecules) in state α and $(1-n)$ would represent the proportion of particles in a β state.

During a depolarizing pulse time varies from t_0 to t_∞ (time at the end of the pulse) and n varies from n_0 to n_∞. The solution of equation (15) is:

$$n = n_\infty - (n_\infty - n_0)\exp(-t/\tau_n) \qquad (16)$$

where $n_\infty = \alpha_n/(\alpha_n + \beta_n)$ and $\tau_n = 1/(\alpha_n + \beta_n)$ and $\alpha_n = n_\infty/\tau_n$ and $\beta_n = (1-n_\infty)/\tau_n$.

The n_∞ values used to determine α_n and β_n are determined from the maximal values of the g_{x1} and g_{x2} conductances determined on semi-logarithmic paper (ordinates at the origin).

The activation curves $i'_{x1}=f(V)$ and $i'_{x2}=f(V)$ were obtained from tail current recordings. The tail currents were plotted on semi-logarithmic scales in a similar manner to Figure 9(f). The i'_{x1} and i'_{x2} values correspond to values determined like I_1 and I_2 (Figure 9f) but obtained from the tail currents (deactivation currents). The ordinates at the origin (i'_{x1} and i'_{x2}) would be representative of the initial state of the deactivation of the delayed current and would be proportional to the activation state n_∞ (at the steady state) of the current components obtained during the preceding stimulus (Noble & Tsien, 1969a).

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PERIPHERAL EFFECTS OF FENFLURAMINE

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1 The peripheral cardiovascular effects of the centrally acting anorexigenic agent, fenfluramine hydrochloride, have been investigated in the rat.

2 After intravenous administration of fenfluramine, an immediate hypotensive response, followed by a reflex rise in blood pressure was recorded. This was followed by a prolonged fall in blood pressure which frequently failed to return to pre-drug levels.

3 The antagonists, propranolol and atropine, failed to inhibit this hypotensive effect of fenfluramine.

4 The effects of 5 mg/kg fenfluramine for 1 h on the blood pressure responses to the sympathomimetic amines, tyramine, methoxamine and metaraminol were studied.

5 The responses to the indirectly acting tyramine were reduced by 50% following fenfluramine, while those to the directly acting methoxamine remained unaffected by the drug. Responses to metaraminol, an amine with both direct and indirect actions, were also unaffected to a significant degree by fenfluramine.

6 Studies on rat isolated vas deferens again showed that responses to tyramine are greatly reduced following fenfluramine.

7 In addition fenfluramine itself produced spontaneous contractions of the vas deferens. These contractions were blocked by the α-adrenoceptor blocking agents phentolamine and thymoxamine.

8 It is suggested that fenfluramine exerts an effect at the adrenergic nerve terminal, either by displacing noradrenaline stores or by inhibition of the amine uptake process.

Introduction

Fenfluramine hydrochloride has been shown to be an anorexigenic agent in animals and man (Le Douarec, Schmitt & Laubie, 1966; Munro, Seaton & Duncan, 1966). The chemical structure of fenfluramine is similar to that of amphetamine, another drug capable of suppressing food intake; however, the introduction of a trifluoromethyl group in the *meta* position on the benzene ring, and an ethyl group on the nitrogen atom of the amphetamine molecule abolishes central stimulant activity (Alphin, Funderburk & Ward, 1964).

Two preliminary investigations into the peripheral effects of fenfluramine have been reported (Franko, Honkomp & Ward, 1965; Sipes, Ziance & Buckley, 1971) and in both cases a hypertensive response to the drug was recorded.

In the brain, fenfluramine produces a long-lasting dose-dependent lowering of 5-hydroxytryptamine concentrations (Duhault & Verdavainne, 1967; Opitz, 1967; Costa, Groppetti & Revuelta, 1971), which, it has been suggested, accounts for the anorexigenic effect of this drug (Jespersen & Scheel-Kruger, 1970; Samanin, Ghezzi, Valzelli & Garattini, 1972). However, a reduction in noradrenaline concentrations following fenfluramine has also been observed both in the brain (Duhault & Verdavainne, 1967) and peripherally (Sipes *et al.*, 1971).

The present study was undertaken to investigate more fully the peripheral effects of fenfluramine. In this context both the direct effects of the drug and the possibility of an indirect response, mediated through sympathetic nerve terminals, were studied.

Methods

Rat blood pressure

Rats, weighing 175–225 g, of either sex, were anaesthetized with urethane (0.75 g/kg i.p. and 0.75 g/kg s.c.). A polythene cannula, inserted into the right carotid artery, was used to measure blood pressure via a Devices transducer and recorder. Drugs were administered through a polythene cannula inserted in the left external jugular vein.

Blood pressure responses to various doses of fenfluramine were recorded for periods up to one hour. Dose–response relationships to various sympathomimetic amines, chosen for their different modes of action, were recorded before and after

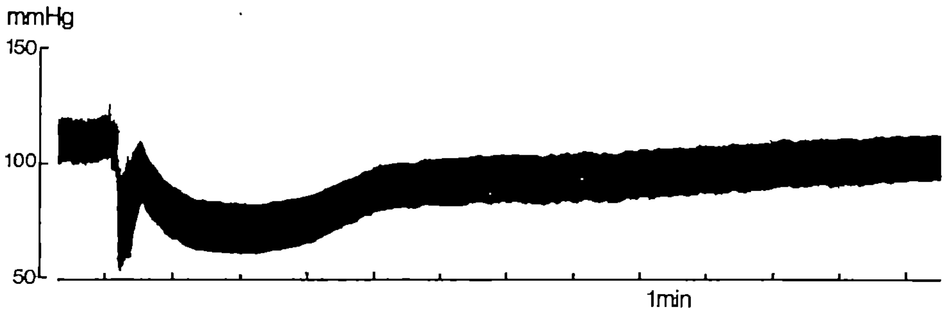

Figure 1 Blood pressure response to fenfluramine hydrochloride 5 mg/kg in the anaesthetized rat.

exposure to 5 mg/kg fenfluramine for one hour.

Drugs were administered in solution in 0.9% w/v NaCl (saline) and volumes administered at any one time did not exceed 0.2 ml.

Rat isolated vas deferens

Male rats, weighing 200–250 g, were killed by a blow on the head. The vasa deferentia were excised, stripped of extraneous material, and suspended in 10 ml organ baths containing Tyrode solution (composition (g/l): NaCl 8.0, KCl 0.2, $MgCl_2$ 0.2, $CaCl_2$ 0.2, NaH_2PO_4 0.05, $NaHCO_3$ 1.0 and glucose 1.0) maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. Isometric contractions were recorded with Devices 2 oz strain gauge transducers and two channel recorder.

Drugs were freshly prepared and administered in Tyrode solution. The following drugs were used: fenfluramine hydrochloride (Ponderax), metaraminol tartrate, methoxamine hydrochloride, tyramine hydrochloride, propranolol hydrochloride, atropine sulphate, phentolamine mesylate and thymoxamine hydrochloride.

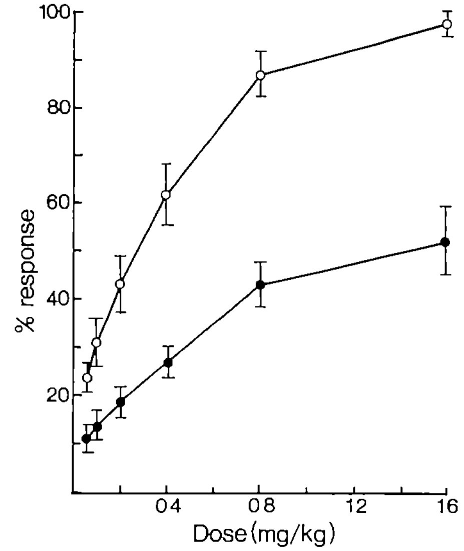

Figure 2 Dose-percentage response relationships for tyramine before (○) and after (●) fenfluramine hydrochloride 5 mg/kg. Responses are measured as the percentage of the maximum control response for each experiment. Each point represents the mean response (± s.e. of the ratio) from 12 experiments.

Results

Direct effects of fenfluramine on rat blood pressure

The recording of a typical blood pressure response to fenfluramine is shown in Figure 1. Fenfluramine 5 mg/kg produced an immediate sharp fall in blood pressure (44 ± 3.6 mmHg, $n = 10$) which was accompanied by respiratory depression. Animals frequently failed to recover following doses of fenfluramine in excess of 10 mg/kg. This hypotensive crisis was followed by a recovery phase, and the blood pressure returned to pre-drug levels. A shallower, but more prolonged phase of hypotension ensued and the blood pressure remained depressed for periods up to 30 minutes. Frequently the blood pressure responses did not revert to pre-drug levels.

Pre-administration of propranolol (up to 1 mg/kg) failed to abolish or reduce the hypotensive effects of fenfluramine. Likewise, atropine (0.5 mg/kg) had no effect on fenfluramine hypotensive responses. Doses of theophylline, up to 5 mg/kg, failed to potentiate the depressor effects of fenfluramine.

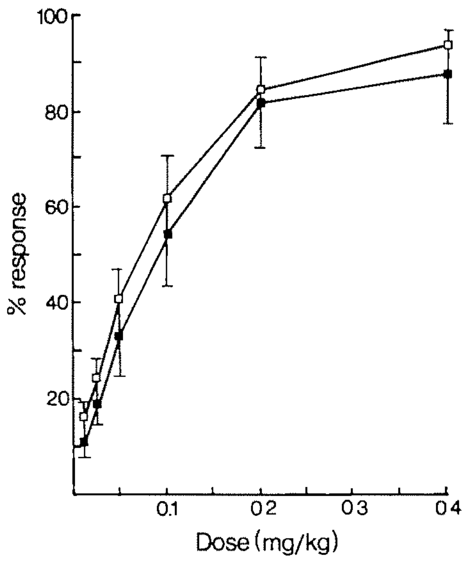

Figure 3 Dose-percentage response relationships for methoxamine before (☐) and after (■) fenfluramine hydrochloride 5 mg/kg. Responses are measured as the percentage of the maximum control response for each experiment. Each point represents the mean response (± s.e. of the ratio) from 8 experiments. Differences in pressor responses before and after fenfluramine are not significant ($P > 0.5$, Student's t test).

Effects of fenfluramine on blood pressure responses to sympathomimetic amines

The sympathomimetic amines, tyramine (indirectly acting), methoxamine (direct) and metaraminol (direct and indirect), were chosen for their different modes of action at adrenergic nerve terminals.

Dose-response relationships were measured for all three amines, before and after exposure of the animals to fenfluramine 5 mg/kg for one hour. Mean results for each drug were plotted graphically. Figure 2 shows that fenfluramine produced a marked reduction in the pressor responses to tyramine. At the dose level of tyramine which produced a maximum control response, fenfluramine produced a reduction in response of approximately 50%. Figure 3 shows the effect of fenfluramine on responses to methoxamine. In this case there was a slight, though not significant reduction in pressor responses following fenfluramine. The effects of fenfluramine on responses to metaraminol are shown in Figure 4. There was a slight increase in the response to the amine following fenfluramine administration. However, the difference in response is not significant for any dose level.

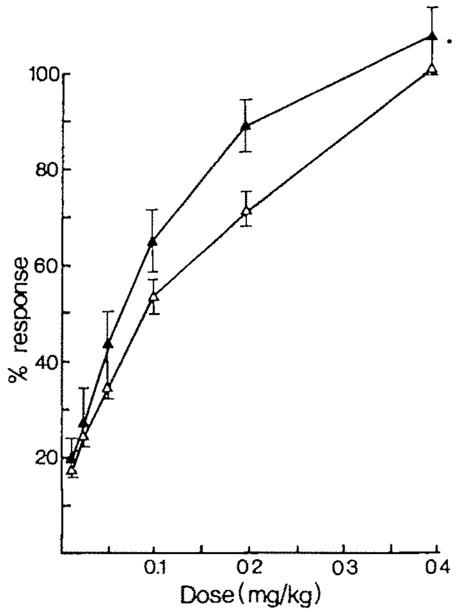

Figure 4 Dose-percentage response relationsips for metaraminol before (△) and after (▲) fenfluramine hydrochloride 5 mg/kg. Responses are measured as the percentage of the maximum control response for each experiment. Each point represents the mean response (± s.e. of the ratio) from 7 experiments. Differences in responses before and after fenfluramine are not significant ($P > 0.05$, Student's t test).

Effects of fenfluramine on tyramine responses in rat isolated vas deferens

Cumulative dose–response relationships to tyramine were obtained in the rat vas deferens before and after exposure to 0.2 mM fenfluramine for one hour. Fenfluramine produced an almost total abolition of tyramine responses (Table 1).

Table 1 Effects of fenfluramine (0.2 mM), for 1 h, on the cumulative dose–response relationship to tyramine in rat isolated vas deferens

Dose of tyramine (μM)	*Control response*	*Response after fenfluramine*
5.0	5.5 ± 0.4	0.0
10	13.0 ± 0.6	0.0
20	25.3 ± 1.6	1.4 ± 0.2
50	53.9 ± 5.0	3.0 ± 0.1
100	88.0 ± 3.8	3.5 ± 0.3
200	99.4 ± 0.7	4.2 ± 0.2

Results are the mean of 6 experiments, and are expressed as mean percentage response ± s.e. of the ratio for each dose of tyramine.

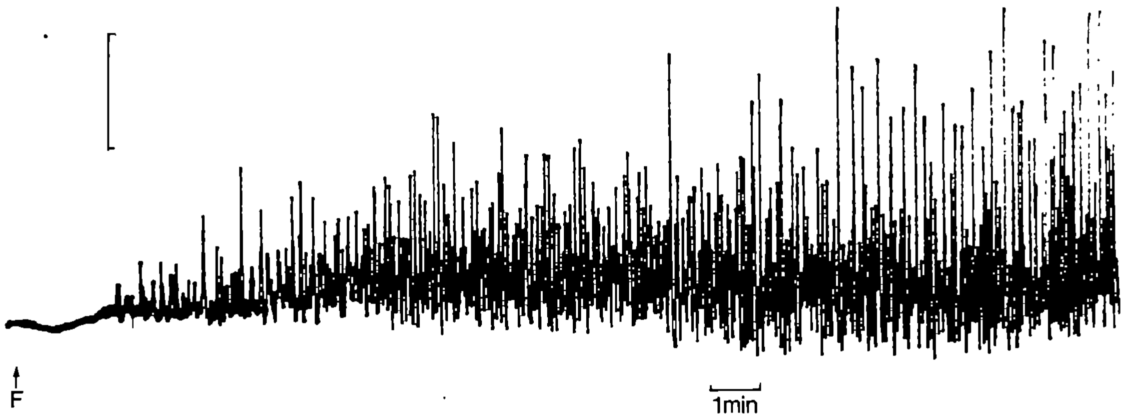

Figure 5 The response of rat isolated vas deferens to 0.2 mM fenfluramine (F). Vertical calibration 2 g, horizontal calibration 1 minute.

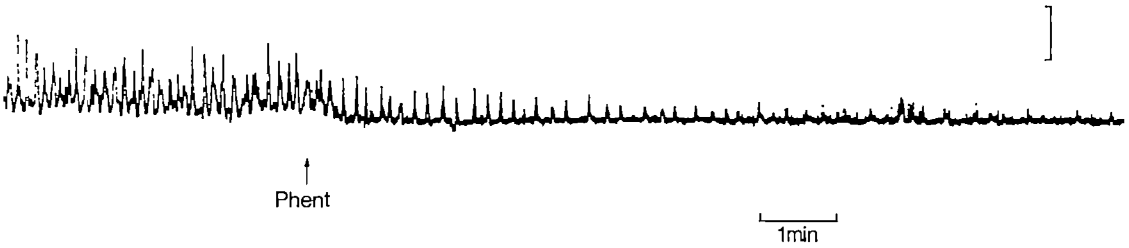

Figure 6 The reduction caused by 50 μM phentolamine (Phent), of contractions of the rat isolated vas deferens produced by 0.2 mM fenfluramine. Vertical calibration 2 g, horizontal calibration 1 minute.

During the period of exposure of the vas deferens to fenfluramine it was noted that the drug produced intermittent but frequent contractions of the tissue (Figure 5); these contractions usually started within 5 min after addition of the drug and persisted for periods in excess of one hour. The amplitude of contraction steadily increased to a maximum which, on average, was equal to the height of contraction produced by a dose of 5 μM noradrenaline.

The spontaneous contractions of vasa deferentia following fenfluramine were abolished by the addition of the α-adrenoceptor blocking agents phentolamine (0.4 mM) or thymoxamine (0.8 mM). Contractions were reduced immediately after the addition of the antagonists, though abolition of the contractions usually occurred 10–15 min after the addition of the drug (Figure 6).

Discussion

Previous work on the effects of fenfluramine on the cardiovascular system indicated that, following an initial hypotensive response, the drug produced a prolonged hypertension (Franko *et al.*, 1965; Sipes *et al.*, 1971). In the present study the initial hypotensive response was followed by a short period of recovery, but this in turn was followed by a further prolonged hypotensive response. This discrepancy may be due to a species difference as the present results were obtained on anaesthetized rats whereas Franko *et al.* and Sipes *et al.* used anaesthetized dogs and cats. If, as results seem to indicate, the more prolonged pressure changes following fenfluramine are mediated by an indirect effect, then the species difference may be due to different modes of action of fenfluramine at the adrenergic terminals of the different animals.

It seems likely that the prolonged effects of fenfluramine are due to an indirect action, but the rapid onset of the initial hypotensive response would suggest that this is due to a direct effect of the drug on a receptor system within the vascular smooth muscle. The possibility of fenfluramine exerting its effect through stimulation of either muscarinic receptors or β-adrenoceptors was therefore considered. However, both atropine and propranolol failed to reduce the degree of hypotension produced by fenfluramine.

To complete the investigation, potentiation by theophylline of the fenfluramine response was studied to establish whether or not fenfluramine had an effect on cyclic adenosine 3′,5′-monophosphate levels through an effect beyond the β-receptor. No such potentiation was seen. It is therefore concluded that fenfluramine produces a hypotensive response through a direct action which is not mediated by stimulation of known autonomic receptor sites.

The three sympathomimetic amines, methoxamine, tyramine and metaraminol, all produced dose-dependent rises in blood pressure through their respective direct, indirect and mixed sympathomimetic modes of action. These responses were affected to a greater or lesser degree by the exposure of the animals to 5 mg/kg fenfluramine for one hour.

The greatest effect was seen with the indirectly acting tyramine and the least affected amine was the directly acting methoxamine. These results suggest that fenfluramine exerts an effect presynaptically at the adrenergic nerve terminal. Biochemical studies (Sipes *et al.,* 1971) on tissue noradrenaline concentrations, following fenfluramine, indicated that the drug releases or displaces noradrenaline from one or more of its neuronal storage sites. The results of the present study lend weight to this theory, since fenfluramine reduced the responses to tyramine by 50% whilst the responses to methoxamine remained unaltered by the drug. It might have been expected that metaraminol, a sympathomimetic amine with both direct and indirect actions, would have been affected to an intermediate degree by fenfluramine. However, the results showed that responses to metaraminol following fenfluramine were increased, though not significantly, over control levels. The possibility that fenfluramine may act as an amine uptake inhibitor at the adrenergic nerve terminal must therefore not be overlooked. This would account for the marked reduction in responses to tyramine and the slight potentiation of responses to metaraminol, since more amine would be available for its direct sympathomimetic effect.

The suggestion that fenfluramine exerts an effect via the sympathetic nerve terminal was confirmed by results on rat isolated vasa deferentia. Fenfluramine produced contractions in the vas deferens, but it seems unlikely that these responses were mediated via a direct effect on fenfluramine-sensitive receptor sites, for a number of reasons. Contractions were not produced immediately following the addition of the drug, as was seen with most agonist–receptor interactions, but periods of up to 5 min elapsed before an effect was seen. The responses themselves were dissimilar to those normally seen following the addition of an agonist being of a phasic appearance and of irregular height indicative of an indirect, packaged release of a transmitter substance. The prolonged period of contractions produced by fenfluramine again suggests, not a direct effect but either an indirect effect or a sustained release of the drug from a storage site. Finally the contractions produced by fenfluramine were abolished by the addition of an α-adrenoceptor blocking agent (phentolamine or thymoxamine).

In conclusion, the results of the present study indicate that fenfluramine produces a prolonged hypotensive response in the anaesthetized rat and that fenfluramine exerts an effect at adrenergic nerve terminals, either by depleting stores of noradrenaline or by inhibiting the amine uptake process or by a combination of the two. However, the effects of the drug on adrenergic terminals and its cardiovascular effects are not necessarily related.

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ANALYSIS OF THE EFFECTS OF *p*-METHOXY-PHENYLETHYLAMINE ON SPINAL CORD NEURONES

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1 Para-methoxyphenylethylamine (PMPEA) was applied microiontophoretically onto interneurones and motoneurones in the spinal cords of acute spinal cats anaesthetized with α-chloralose. Its effects were compared with those of noradrenaline (NA) and 5-hydroxytryptamine (5-HT).

2 PMPEA had effects on interneurones which were similar to those of NA and/or 5-HT; its action was predominantly depressant, and it rarely affected interneurones which could not be influenced by NA or 5-HT.

3 The actions of PMPEA on interneurones excited by electrical stimulation of leg nerves showed that the population of interneurones influenced by the drug coincides with the population affected by NA and 5-HT and by intravenously administered PMPEA.

4 Renshaw cells, which are excited by intravenous PMPEA, were either not affected or depressed by microiontophoretically applied PMPEA.

5 Alpha motoneurones, which are depolarized by intravenous PMPEA, were hyperpolarized by microiontophoretically applied PMPEA. Antidromic firing of the cells could be blocked by PMPEA.

6 The differences between the effects of intravenous infusion and the iontophoretic application of PMPEA upon motoneurones is most easily explained by inhibition of interneurones and a concomitant disinhibition of motoneurones. A similar mechanism may also account for the different effects seen with intravenous and iontophoretic application of PMPEA on Renshaw cells.

Introduction

The effects of intravenously administered *p*-methoxyphenylethylamine (PMPEA) on the cat spinal cord have been previously described (Walker, Willis & Willis, 1970; Jordan, Willis & Matthews, 1972; Jordan & Willis, 1973; Willis, Ashkenazi, Willis & Haber, 1973). In doses of 2.5 mg/kg or greater it enhances monosynaptic reflexes of both flexor and extensor motoneurones (Walker *et al.*, 1970), and this reflex enhancement is accompanied by depolarization of the membrane potentials of α-motoneurones and by decreased polysynaptic excitatory and inhibitory polysynaptic potentials recorded from motoneurones (Jordan *et al.*, 1972). It also interferes with transmission in interneuronal pathways in the spinal cord, it depresses the firing of single interneurones in the spinal cord grey matter, and it reduces synaptic noise in motoneurones (Jordan & Willis, 1973).

These actions of intravenously administered PMPEA are due to a central action of the drug on the spinal cord (Walker *et al.*, 1970), and various lines of evidence suggest that these effects on central nervous system neurones are due to an action on monoaminergic synapses. It is a sympathomimetic agent (Epstein, Gunn & Virden, 1932), and its hypertensive action, like that of tyramine, can be reduced by reserpine or guanethidine pretreatment, suggesting that it causes release of noradrenaline (NA) from nerve terminals (Cession-Fossion & Michaux, 1963). Paton & Pasternak (1974) have shown that PMPEA accelerates the efflux of NA from atria, and Horn (1973) showed that it may cause inhibition of catecholamine uptake. PMPEA also blocks the uptake by isolated synaptosomes of NA and 5-hydroxytryptamine (5-HT) (Ashkenazi, Haber, Coulter & Willis, 1973), and it can reduce the levels of both NA and 5-HT in the spinal cord of mice (Ashkenazi, Willis & Haber, 1974). The effects of PMPEA on reflexes can be antagonized by blockers of α-adrenoceptors and tryptamine receptors (Walker *et al.*, 1970), and its actions on monosynaptic reflexes, on excitatory and inhibitory interneuronal pathways to motoneurones, and on the pathways causing primary afferent depolarization are similar to those of monoamine precursors and other substances which increase monoamine levels in the spinal cord (for references, see Jordan *et al.*, 1972 and Jordan & Willis, 1973). The enhancement of reflexes produced by PMPEA is potentiated by the monoamine precursors 5-hydroxytryptophan (5-HTP) and 3,4-dihydroxyphenylalanine (L-DOPA) and by the monoamine oxidase inhibitor, nialamide (Coulter, Bird, Willis & Willis, 1972; Willis *et al.*, 1973). All of these studies strongly support the notion that PMPEA acts at noradrenergic as well as 5-hydroxytryptaminergic synapses in the spinal cord.

Previous studies conducted with intravenously administered PMPEA have not revealed whether its actions on motoneurones and interneurones are due to direct postsynaptic effects or to release of monoamines from terminals within the spinal cord, nor have they shown whether the effects of PMPEA are limited to monoamine synapses. Furthermore, it is necessary to reconcile the depolarizing effect of intravenously administered PMPEA on motoneurones with the finding that microiontophoretically applied NA and 5-HT cause hyperpolarization of these cells (Phillis, Tebečis & York, 1968; Engberg & Thaller, 1970; Engberg & Marshall, 1971). Accordingly, the effects of microiontophoretically applied PMPEA on spinal cord interneurones, Renshaw cells and motoneurones have been determined and compared with those obtained with NA and 5-HT. A preliminary report of some of these results has been presented (Jordan, 1972).

Methods

Twenty-six cats were used in these experiments. The animals were anaesthetized with α-chloralose (80 mg/kg, i.v.) and the spinal cord was transected at the level of L1. After laminectomy, the L6, L7 and S1 ventral roots were cut, and the central end of L7 or S1 was mounted for stimulation on bipolar platinum electrodes. The leg nerves posterior biceps-semitendinosis (PBST), semimembranosis-anterior biceps (SMAB), gastrocnemius-soleus (GS), tibalis anterior (TA), and sural (S) were cut, and the central ends were mounted on bipolar platinum electrodes for stimulation. Constant current stimuli of 0.1 ms duration were applied with a Grass S88 stimulator and a Grass Constant Current unit. Carotid artery blood pressure was continuously recorded on a penwriter, and rectal temperature was maintained at 37°C using a heating pad and a feed-back circuit.

Seven-barrelled micropipettes with tip diameters of 4–8 µm were used for recording extracellular action potentials and for administering drugs microiontophoretically. The central recording barrel contained 2M NaCl, while the surrounding barrels were filled by centrifugation with various combinations of the following solutions: noradrenaline bitartrate (Sigma; 0.2M, pH 5.0), 5-hydroxytryptamine creatinine sulphate (Sigma; 0.04 M, pH 6.0), *p*-methoxyphenylethylamine hydrochloride (Calbiochem, 0.2 M, pH 4.5), L-glutamate, monosodium salt (Sigma; 0.2 M), acetylcholine chloride (Sigma, 0.2 M), and NaCl (Baker; 2 M). Drugs (except glutamate) were ejected as cations by passing anodal currents from constant current sources (Spencer, 1971) through the microelectrodes. Cations were prevented from diffusing from the drug barrels by applying cathodal retaining currents of 8–10 nA, whereas glutamate was retained by passage of anodal currents and ejected by passing cathodal currents. An effect of a drug on neuronal firing rate was judged to be genuine if recovery occurred, if the effect was repeatable, and if it was not mimicked by a current control. A drug was judged to have no effect if a 1 min application at a current of 150 nA or more failed to produce a change in firing rate or membrane potential. Cationic current controls were performed by passing Na^+ from a barrel filled with NaCl.

When intracellular recording with simultaneous extracellular drug applications was attempted, three-barrelled microelectrodes for extracellular recording and drug applications were positioned in Plexiglass holders similar to that described by Phillis *et al.* (1968). Intracellular records were achieved through the use of an attached fine-tipped microelectrode (filled with 2 M KCl or 3 M NaCl), which fitted into a groove between two adjacent barrels of the multi-barrelled microelectrode and projected 20–60 µm beyond the tips of the drug-containing micropipettes. The arrangement allowed simultaneous drug application and intracellular as well as extracellular recording. The intracellular recording barrels had resistances of 10–20 megohms. Resting potentials were observed with a d.c.-coupled amplifier and were recorded by photographing the oscilloscope screen at 3 s intervals.

Extracellularly recorded potentials were amplified and displayed on an oscilloscope for photography. Effects of drugs on synaptically evoked firing were determined from filmed records. Spontaneous and glutamate-evoked firing rates were recorded through the use of a window discriminator and an electronic counter (Hewlett-Packard 5214L). Only units which could be clearly distinguished from background noise were accepted for analysis. The output of the counter was converted to analogue form and displayed on a pen recorder. Effects of drugs on firing rate were determined from the pen recorder records.

The synaptically-evoked firing of interneurones and motoneurones, as well as antidromic firing produced in motoneurones, was recorded by photographing the oscilloscope screen. Interneurones were identified by their failure to respond to ventral root stimulation and by their characteristic discharge of multiple action potentials in response to a single stimulus applied to a peripheral nerve. Motoneurones and Renshaw cells were identified by their response to ventral root stimulation.

A ball-tipped silver–silver chloride electrode was placed at the dorsal root entry zone and was used to detect the arrival at the spinal cord of afferent volleys evoked by leg nerve stimulation. Interneurones which

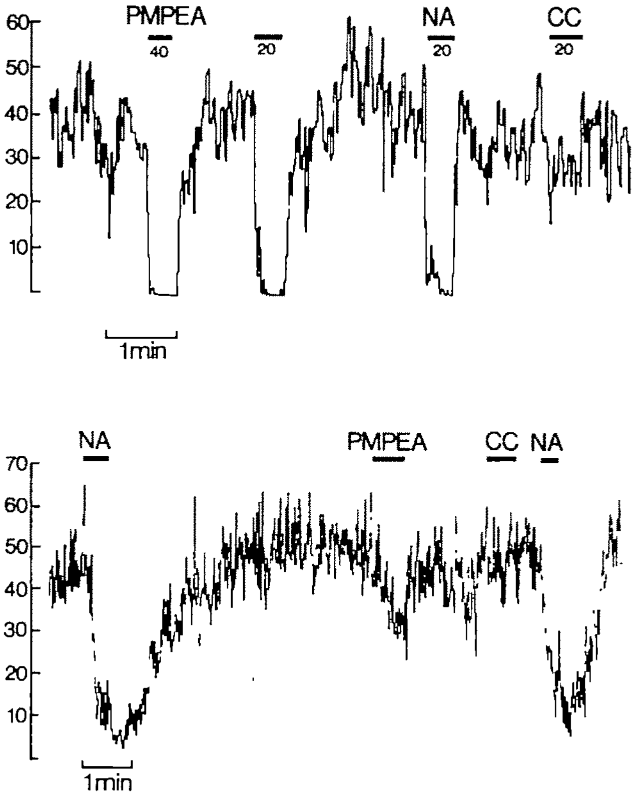

Figure 1 The effects of *p*-methoxyphenylethylamine (PMPEA) and noradrenaline (NA) on spontaneous firing of two spinal interneurones. The ordinates represent firing rate in spikes per second. The firing of the neurone illustrated in the upper trace was depressed by applications of PMPEA of 40 and 20 nanoamperes (nA) during the periods indicated by horizontal bars. The effects of NA (20 nA) and current control (CC) are also illustrated. In the lower trace, the responses to drugs and current control (100 nA in all cases) are illustrated.

fired a spike within 0.5 ms after the arrival of the afferent volley at the surface of the spinal cord were considered to be monosynaptically activated. Stimulus strengths required for activation of low threshold and high threshold afferent fibres in leg nerves were determined from the cord dorsum record; stimulus strengths which produced the first detectable negative waves on the cord dorsum record were considered adequate for exciting low threshold afferent fibres. Effects which could be produced only by stimulus strengths more than ten times threshold for cord dorsum evoked potentials were considered to be due to activity in high threshold afferents.

Results

Effects of microiontophoretically applied p*-methoxyphenylethylamine, noradrenaline and 5-hydroxytryptamine on interneurones*

PMPEA was ejected microiontophoretically in the vicinity of 71 interneurones in the lumbar enlargement. The drug was tested on spontaneous firing of 30 of these; 35 were excited by stimulation of peripheral nerves (on three of the latter PMPEA was also tested on spontaneous firing). Figure 1 illustrates the effects of PMPEA and NA on two different spontaneously firing interneurones. NA had a powerful depressant action on both of these neurones, and PMPEA also depressed them. The reduction in firing rate caused by these drugs was not mimicked by control currents passed from an adjacent barrel containing NaCl. In general, PMPEA either had no effect on cells influenced by NA or 5-HT, or its effects were qualitatively similar to those of the monoamines. PMPEA was nearly always without effect on cells that were not influenced by NA or 5-HT. Table 1 shows the distribution of effects obtained with NA, 5-HT and PMPEA. These findings suggest that the action of PMPEA on spinal interneurones is primarily one of depression, and that its effects on interneurones may be dependent upon the presence of monoamine receptors. Table 2 is a comparison of the effects of PMPEA with those of NA and/or 5-HT on the same

Table 1 Effects of *p*-methoxyphenylethylamine (PMPEA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) on spontaneously firing, glutamate-activated and orthodromically activated spinal cord interneurones

	% Excited	*% Depressed*	*% Unaffected*
Orthodromically activated			
PMPEA (35)*	0	11	89
NA (50)	4	32	64
5-HT (34)	2	19	79
Spontaneously firing†			
PMPEA (30)	3	40	57
NA (41)	12	56	32
5-HT (18)	5	39	56
Glutamate-activated			
PMPEA (9)	0	100	0
NA (19)	0	68	32
5-HT (7)	0	86	14

* Numbers in brackets indicate total number of cells tested with each drug in each category; † 3 cells in this category were also tested using orthodromic activation.

Table 2 Comparison of the effects of *p*-methoxyphenylethylamine (PMPEA) with those of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) on the same cells

PMPEA		*NA*			*5-HT*		
		No. excited	*No. depressed*	*No. unaffected*	*No. excited*	*No. depressed*	*No. unaffected*
No. excited	2	1	1	0	1	0	0
No. depressed	24	0	20	1	0	12	3
No. unaffected	41	4	12	22	1	2	25

Table 3 Effects of *p*-methoxyphenylethylamine (PMPEA) on orthodromically activated spinal cord interneurones

Source of excitation		*PMPEA*	
	No. excited	*No. depressed*	*No. unaffected*
Monosynaptically excited from low threshold muscle afferents (2)*	0	0	2
Monosynaptically excited from high threshold muscle afferents (3)	0	1	2
Polysynaptically excited from high threshold muscle afferents (5)	0	1	4
Polysynaptically excited from skin afferents (10)	0	0	10
Polysynaptically excited from skin afferents and high threshold muscle afferents (15)	0	2	13

* Numbers in brackets indicate the number of neurones in each category.

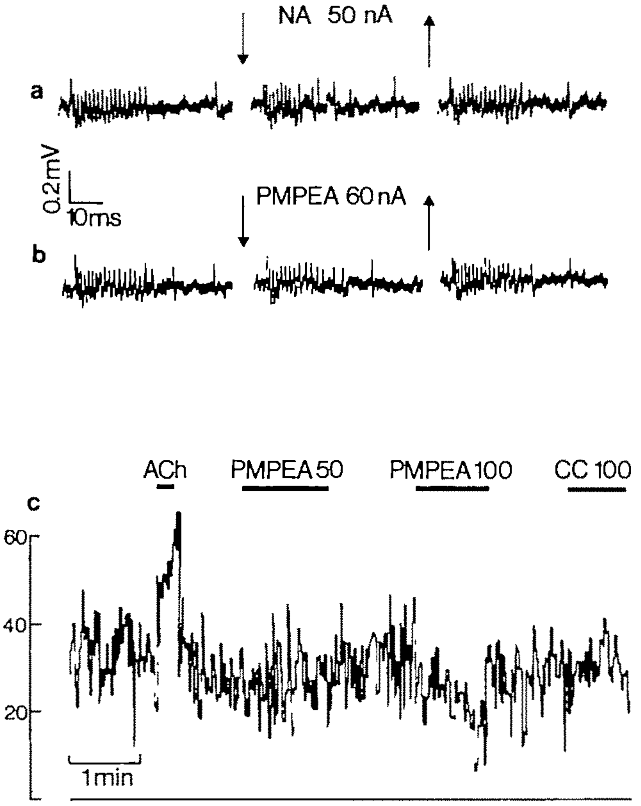

Figure 2 The actions of noradrenaline (NA), acetylcholine (ACh) and *p*-methoxyphenylethylamine (PMPEA) on Renshaw cells: (a) and (b) are photographs of oscilloscope traces of burst discharges produced by ventral root stimulation. Drug applications occurred between the arrows. No effect was observed when a subsequent current control was applied. (c) is a ratemeter record of spontaneous firing of a second neurone. The ordinate represents spikes per second. Drug application occurred during the time period indicated by the horizontal bars. ACh was applied using a current of 15 nA.

interneurones. It can be seen that the neurones which were depressed by PMPEA were also consistently depressed by NA and/or 5-HT. Only 1 of 24 interneurones depressed by PMPEA and also tested with NA or 5-HT was uninfluenced by NA or 5-HT.

In agreement with Engberg & Ryall (1966), it was found that firing of interneurones produced by orthodromic activation of afferents in leg nerves was more difficult to influence by microiontophoretic applications of NA and 5-HT than spontaneous or glutamate-evoked firing (Table 1). The same was true for PMPEA, which depressed only 4 of 35 orthodromically excited interneurones and had no effect on the remainder.

Table 3 summarizes the effects of PMPEA on orthodromically excited interneurones. It can be seen that the interneurones depressed by PMPEA were excited by stimulation of skin and high threshold muscle afferents (flexion reflex afferents). This population of interneurones coincides with that depressed by intravenous infusion of PMPEA (Jordan & Willis, 1973).

The effects of NA, 5-HT and PMPEA were determined on 10 Renshaw cells identified by their response to antidromic stimulation of the ventral root. The effects of NA and PMPEA on synaptically evoked firing and of NA, acetylcholine (ACh) and PMPEA on spontaneous firing of Renshaw cells is illustrated in Figure 2. NA and PMPEA reduced the number of action potentials produced in response to ventral root stimulation (Figure 2a and b). Figure 2c shows the response of a spontaneously firing Renshaw cell to ACh and PMPEA. This cell was markedly excited by ACh, showing that the firing rate was sufficiently low to allow development of an excitatory response. Nevertheless, PMPEA had only a mild depressant action on this cell. PMPEA depressed the spontaneous or synaptically evoked firing of 6 of the

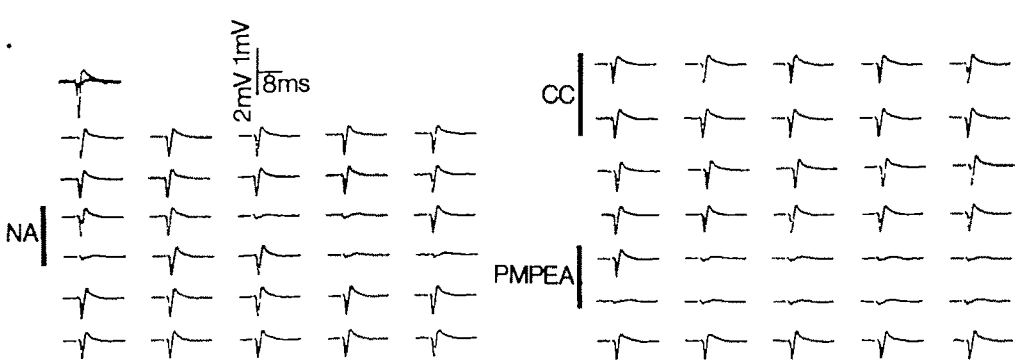

Figure 3 Effects of *p*-methoxyphenylethylamine (PMPEA) and noradrenaline (NA) on antidromic firing of a motoneurone. Stimulation of the L7 ventral root at threshold (2 superimposed traces at upper left) revealed the all-or-nothing nature of the spike (negative downwards). The 1 mV calibration applies to this record. Subsequent traces at lower gain are consecutive records reading from left to right taken at 3 s intervals. Drugs and control current (CC) were applied during the periods indicated by vertical bars. Currents of 100 nA were used in each case. These effects were obtained using a multibarrelled extracellular electrode.

10 Renshaw cells studied and had no effect on the remaining 4. NA depressed 5 of the same 10 cells and had no effect on the remainder. 5-HT was tested on 7 Renshaw cells, having no effect on 5 and a depressant action on 2. ACh always excited the Renshaw cells on which it was tested. The effects obtained with NA and 5-HT are in agreement with the depressant action of NA on these cells reported by Engberg & Ryall (1966). The fact that PMPEA can mimic these effects suggests that the drug may interact with the same receptors as those for NA or 5-HT. The failure of microiontophoretically applied PMPEA to produce excitation, however, is not similar to the effects obtained on recurrent inhibition and Renshaw cell discharge observed when PMPEA is infused intravenously (Jordan & Willis, 1973).

Effects of p-*methoxyphenylethylamine and noradrenaline on α-motoneurones*

Since PMPEA routinely causes depolarization of motoneurones when infused intravenously, it was of interest to determine the direct effect of PMPEA on motoneurones and to compare it with the well documented hyperpolarizing action of NA on these cells (Phillis *et al.*, 1968; Engberg & Thaller, 1970; Engberg & Marshall, 1971). The effects of these drugs on extracellularly recorded firing of motoneurones were determined. Motoneurones were identified by the presence of action potentials with discrete thresholds to antidromic stimulation of ventral roots which followed stimulation at high rates (up to 200/second).

Figure 3 shows the type of response caused by NA on motoneurone firing produced by peripheral nerve or ventral root stimulation. In agreement with Weight & Salmoiraghi (1967), the extracellularly-recorded spikes can be blocked by NA. NA blocked antidromic firing in 5 of 11 motoneurones and had no effect on the remainder. Figure 3 also shows the actions produced by PMPEA on extracellularly recorded motoneurone antidromic action potentials, indicating that microiontophoretic application of PMPEA, unlike intravenous infusions of the drug, causes depression of motoneurone discharges. PMPEA was applied near 14 motoneurones from which extracellular spikes were recorded; in 4 of these neurones the spikes were blocked during the action of PMPEA, whereas application of PMPEA with currents up to 150 nA had no effect on the remaining 10 neurones. PMPEA never caused firing or increased excitability of motoneurones, regardless of ejecting current.

Intracellular records were obtained from over 60 motoneurones with extracellular microiontophoretic application of NA and/or PMPEA. Initial resting potentials ranged from 44 to 72 mV. Three-barrelled drug electrodes usually contained NA, PMPEA and NaCl. The NaCl barrel could be used for applications of control currents as well as for extracellular recording. The proximity of the drug electrode to the impaled motoneurones could be assessed by observing the simultaneously recorded intracellular as well as extracellular actions potentials. The intra- and extracellular electrodes were judged to be recording from the same neurone if the action potentials recorded from both had identical thresholds, latencies, and ability to follow high frequency stimulation. Changes in membrane potential were judged to be genuine effects of the drug if they occurred in cells with stable membrane potentials, if they recovered fully after drug application was terminated, and if the effect was not mimicked by a positive control current applied through the extracellular NaCl barrel.

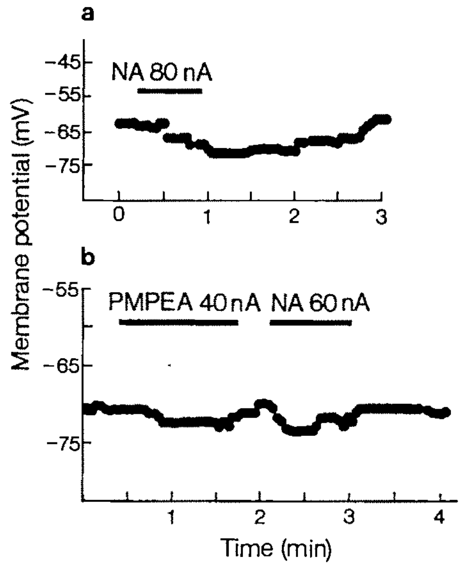

Figure 4 Resting membrane potential changes in motoneurones resulting from noradrenaline (NA) and *p*-methoxyphenylethylamine (PMPEA) application. Each dot represents the value of the membrane potential measured from photographs of oscilloscope traces taken at 3 s intervals. Drug applications are represented by horizontal bars.

Figure 4 shows examples of resting membrane potential changes produced by NA and PMPEA. The hyperpolarization produced by NA is consistent with effects on motoneurone membranes previously reported (Phillis *et al.*, 1968; Engberg & Thaller, 1970; Engberg & Marshall, 1971). The average peak hyperpolarization produced in 5 motoneurones by NA was 4 mV (range 2–9 mV). In three other motoneurones with stable resting potentials NA was without effect when applied at currents up to 150 nA. In the remainder of the motoneurones studied in this way, depolarization of the neurone during drug application occurred which led progressively to loss of the cell, presumably due to damage of the membrane by the electrode. In only one of these cells could even partial recovery be observed.

The effects of PMPEA were observed on 12 motoneurones which had stable resting potentials before and after drug application and in which membrane changes produced by the drug recovered fully after termination of drug application. Hyperpolarization was observed in 3 motoneurones, and one of these effects is shown in Figure 4. NA also hyperpolarized this motoneurone. The average hyperpolarization produced by PMPEA was 8 mV (range 2–16 mV). PMPEA (currents up to 150 nA) had no influence on 9 motoneurones with stable resting potentials. In a number of motoneurones PMPEA application was followed by depolarization of the cell which did not recover. In only one of these was partial recovery observed, possibly due to improvement of the recording conditions. Microionotophoretically applied PMPEA was never observed to cause depolarization of motoneurones which resembled that produced by intravenously infused PMPEA (Jordan *et al.*, 1972), indicating that the latter effect cannot be due to direct depolarization of motoneurones by PMPEA.

Discussion

The results of these experiments reveal the type of direct actions PMPEA exerts on spinal cord neurones of various types, and they allow some conclusions to be drawn about the means whereby intravenous PMPEA influences spinal mechanisms. In general, microiontophoretic application of PMPEA reveals that the drug either has no effect on spinal neurones or it depresses them. The findings that the direct action of PMPEA on spinal interneurones is primarily one of depression, and that it tends to occur only on neurones which can also be depressed by monoamines, suggest that the depression of interneurones produced by PMPEA regardless of the route of administration is dependent upon the presence of monoamine receptors on the affected neurones.

This is consistent with the finding that microiontophoretically applied 3,4-dimethoxyphenylethylamine and PMPEA usually had no effect on caudate neurones which were not influenced by dopamine (Zarzecki, 1974). These results are also consistent with the depression of interneurones, motoneurone synaptic noise and transmission in polysynaptic pathways in the spinal cord observed when PMPEA is infused intravenously (Jordan *et al.*, 1972; Jordan & Willis, 1973). They do not, however, reveal whether the depressions produced by PMPEA are due to direct effects of the drug on interneurone postsynaptic membranes or to release of monoamines from nearby noradrenergic or 5-hydroxytryptaminergic presynaptic terminals.

The interneurone population depressed by microiontophoretically applied PMPEA, so far as it has been identified, coincides well with the population influenced by NA (Engberg & Ryall, 1966) and by intravenous PMPEA (Jordan & Willis, 1973). The depressant effects of NA, 5-HT and PMPEA, when they were observed on identified neurones (orthodromically activated neurones, Table 1), were limited to interneurones activated by flexion reflex afferents. Thus, the same population of interneurones seem to be influenced by intravenous PMPEA as well as by microiontophoretically applied PMPEA and monoamines. This suggests that the mechanisms of action of microiontophoretically applied and intravenously administered PMPEA on interneurones are identical, and that PMPEA selectively influences monoamine synapses on interneurones.

The response of Renshaw cells to microiontophoretically applied PMPEA also resembled the effects obtained with NA and 5-HT on these cells. This depressant action of PMPEA does not resemble the response to intravenously administered PMPEA, however, which was one of enhancement of the burst discharge in response to antidromic stimulation (Jordan & Willis, 1973). It is likely, therefore, that the effect of intravenously administered PMPEA on Renshaw cells is not due to direct effects on these neurones, but is the result of an action on other cells. This could occur subsequent to increased motoneurone activity resulting from the depolarization in motoneurones caused by intravenous PMPEA (Jordan *et al.*, 1972), or it could arise from removal of inhibitory influences from other interneurones (cf. Wilson, Talbot & Kato, 1964) which may be depressed by NA, 5-HT or PMPEA. Although occasional excitant actions of NA on Renshaw cells have been observed in studies using the method of microiontophoresis (Weight & Salmoiraghi, 1965; Engberg & Ryall, 1966), no such excitant actions were observed in this study. In a subsequent study by Weight & Salmoiraghi (1966), a predominance of depressant actions were observed when NA was tested on Renshaw cell firing.

The experiments on the effects of microiontophoretically applied PMPEA on motoneurone excitability and transmembrane potentials clearly show that the depolarization of motoneurones and the enhancement of monosynaptic reflexes produced by intravenous PMPEA cannot be attributed to a direct postsynaptic depolarizing effect of the drug. As was the case with interneurones, the effects of directly applied PMPEA parallel those of NA. It is likely, therefore, that intravenously administered PMPEA facilities monosynaptic reflex transmission and depolarizes motoneurone membranes through an inhibition of interneurones and a concomitant disinhibition of motoneurones, as suggested previously (Jordan & Willis, 1973). The excitation of motoneurones by intravenous PMPEA through effects on interneurones must be more powerful than any direct depressant action the drug might have on motoneurones. The fact that L-DOPA (Baker & Anderson, 1970) and 5-HTP (Andén, Jukes & Lundberg, 1964) cause excitation of motoneurones, presumably due to widespread release of monoamines in the spinal cord, supports the notion that monoamines must have a more powerful effect on motoneurones by way of actions on interneuronal pathways than through actions at monoamine synapses on motoneurones. It seems likely that microiontophoretically applied PMPEA hyperpolarizes motoneurones by an action similar to the hyperpolarizing actions of microiontophoretically applied NA and 5-HT (Phillis *et al.*, 1968; Engberg & Thaller, 1970; Engberg & Marshall, 1971; 1973). This action of PMPEA might be due to an interaction with monoamine receptors on motoneurones. It is also conceivable that PMPEA might hyperpolarize motoneurones due to an interaction with monoamine terminals on motoneurones. Furthermore, it is possible that PMPEA might hyperpolarize motoneurones due to disfacilitation (cf. Hubbard, Llinas & Quastel, 1969). The drug might reduce excitatory input by an action on nearby interneurones, or by reducing the release of excitatory transmitters from synapses on motoneurones, as suggested by Krnjević (1974) as a possible explanation for the hyperpolarizing action of NA.

In order to establish transmitter roles for monoamines in the spinal cord, it is necessary to show that release of NA and 5-HT from the descending monoaminergic pathways produces the same effects on the neurones of the spinal cord as those produced when exogenous monoamines are applied. Experiments with monoamine precursors have failed to establish this, since the effects of L-DOPA (Andén, Jukes & Lundberg, 1966; Baker & Anderson, 1970) and of 5-HTP (Shibuya & Anderson, 1968) on spinal cord activity are not reduced after chronic transection of the spinal cord. Engberg, Lundberg & Ryall (1968) have shown, however, that the action of reserpine after pretreatment with nialamide (i.e., reduced transmission in flexor reflex afferent pathways) is no longer present in chronic spinal cats. The similarity of the effects of PMPEA on flexor reflex afferent pathways (Jordan *et al.*, 1972; Jordan & Willis, 1973) to those of reserpine suggest that PMPEA may have an action on endogenous monoamine stores. This is also suggested by the finding that PMPEA can accelerate the efflux of NA (Paton & Pasternak, 1974) and cause depletion of both NA and 5-HT (Ashkenazi *et al.*, 1974).

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COMPOUNDS DESIGNED TO FIT A SITE OF KNOWN STRUCTURE IN HUMAN HAEMOGLOBIN

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1 The three-dimensional coordinates of the atoms in human haemoglobin are known, and there is a specific site in the deoxygenated form of the protein at which 2,3-diphosphoglycerate (DPG) interacts.

2 Molecular models of this site have been constructed and used to design compounds which should bind to the deoxy conformation and stabilize it. These compounds should thereby promote oxygen liberation, as does DPG.

3 The compounds so designed were found to promote oxygen liberation. Their relative potencies, as assessed by sigmoidal dose-response curves, are in the predicted sequence.

Introduction

It has long been accepted (Langley, 1878) as a working hypothesis that some drugs may interact with specific receptors of complementary structure. Attempts have been made to deduce the properties and structures of such receptors from the properties of the interacting drugs (e.g. Clark, 1937; Belleau, 1967; Smythies, 1975). There are also direct biochemical observations on receptors suggesting, for example, that the membrane-bound acetylcholine receptor from *Torpedo electroplax* is a protein (Eldefrawi & Eldefrawi, 1973). Similarly, the cardiac glycoside receptor site in mammalian cell membranes may be situated on a tetrameric protein (Stein, Lieb, Karlish & Eilam, 1973). Haemoglobin is also a tetrameric protein, and has been singled out as a model of unique value for the study of drug actions (Clark, 1937).

Detailed knowledge of the structure of any receptor had to await the advent of high-resolution X-ray crystallography. Sperm whale myoglobin was the first biological macromolecule for which the three-dimensional positions of individual atoms were established (Kendrew, Watson, Strandberg, Dickerson, Phillips & Shore, 1961). The molecular structure of horse haemoglobin was determined by Perutz, Muirhead, Cox, Goaman, Mathews, McGandy & Webb (1968). The haemoglobin tetramer contains two α and two β subunits, each consisting of a single polypeptide chain which resembles one myoglobin molecule.

There is a site of known structure on the human deoxyhaemoglobin tetramer at which the small molecule 2,3-diphosphoglycerate (DPG) binds and stabilizes the deoxy conformation. The resulting liberation of oxygen can be measured to provide a quantitative biological response. Thus the DPG-haemoglobin interaction has some characteristics of a 'drug-receptor system', and human haemoglobin was therefore selected as a model pharmacological receptor for the present study. The appropriateness of this choice cannot yet be fully assessed. It is open to the criticism that the haemoglobin molecule is not subject to the same constraints and interactions with neighbouring molecules as a membrane-bound pharmacological receptor. Haemoglobin is also present in greater amounts by several orders of magnitude. On the other hand the choice offers a number of advantages. Haemoglobin can be prepared in a relatively pure form, and its structure and function are well established as is the mode of action of DPG.

Greenwald (1925) found DPG to be present in erythrocytes in millimolar concentrations, but the function of these large amounts remained unknown for 40 years although Sutherland, Posternak & Cori (1949) discovered that micromolar concentrations were needed in order to catalyze the phosphoglyceromutase reaction. In 1963 Sugita & Chanutin observed electrophoretically that DPG formed reversible complexes with haemoglobin, and Chanutin & Curnish (1967) showed that it altered the dissociation curve of haemoglobin, decreasing the oxygen affinity. At about this time Monod, Wyman & Changeux (1965) published their theory of macromolecular properties citing haemoglobin as an example, and Benesch & Benesch (1967) proposed that DPG complexed with haemoglobin as an allosteric regulator. Finally Arnone (1972) obtained crystals of deoxyhaemoglobin which incorporated DPG, and showed by X-ray crystallography how this compound fitted its receptor site in the protein.

A scale model (Beddell, Goodford, Norrington &

Wilkinson, 1973) of the complete haemoglobin tetramer has been constructed as the basis for designing compounds to fit the DPG site. It was predicted that such compounds, like DPG, would diminish the affinity of haemoglobin for oxygen, and their specific effects on the dissociation curve are now described.

Methods

Preparation of human haemoglobin

Human haemoglobin of low phosphate content was prepared by counterflow dialysis (Paterson, Eagles, Young & Beddell, 1976). All preparative work was conducted at 4°C. Red blood cells from 2–5 weeks old blood were washed with 0.9% w/v NaCl solution (saline) and haemolysed in distilled water. The red cell ghosts were removed by centrifugation, and the proteins were fractionated by ammonium sulphate precipitation. Haemoglobin precipitated between 33%–65% saturation, and this precipitate was redissolved and dialysed using a sterilized hollow cellulose fibre dialyser (Dow Chemical Company, Miniplant C/HFD-15). The dialysing buffer was passed through the fibres, and the surrounding solution was analysed for total haemoglobin, methaemoglobin and phosphate after 48 hours. Separate aliquots were stored sterile at −20°C.

Determination of the dissociation curves

The method of Allen, Guthe & Wyman (1950) was modified as follows: 5 cm^3 of the test solution was placed in a cylindrical all-glass tonometer of approximately 100 cm^3 capacity to one end of which was fused a spectrophotometer cell. The air pressure was reduced to 8 kPa while a small flow of nitrogen (British Oxygen Company oxygen-free 'white spot' nitrogen) was admitted. Lower pressures induced frothing and were therefore avoided. Atmospheric pressure was then restored by readmission of nitrogen, and the tonometer was rotated horizontally for 3 min in a water bath at 37°C while the thin film of test solution on the tonometer walls equilibrated with the gas phase. The tonometer was next placed upright in the sample position of a thermostated Pye-Unicam SP 800 spectrophotometer, and the spectrum from 500 to 650 nm was observed. After three or four successive evacuations followed by readmission of nitrogen a constant spectrum of deoxyhaemoglobin was recorded. A small measured volume of air was admitted to the tonometer through a 'Rotaflow' tap, equilibrated with the test solution as before, and a second spectrum observed. After successive readmissions of air a series of spectra were generated which were completed by a final observation of the test solution in pure oxygen. Measurement of each spectrum allowed the proportions of oxy and deoxy haemoglobin to be calculated, and the corresponding oxygen pressure was determined from the total volume of air admitted. Appropriate corrections were applied, and the results were expressed as a dissociation curve showing the proportion of oxyhaemoglobin as a function of oxygen pressure.

Curve fitting

The method of Powell (1970) was used to provide iterative least squares fits to the observations. The equation of Monod *et al.* (1965):

$$Y = \frac{Lc\alpha(1+c\alpha)^3 + \alpha(1+\alpha)^3}{L(1+c\alpha)^4 + (1+\alpha)^4}$$

was used to fit the dissociation curves (e.g. in Figure 2), where L and c are the fitted constants, α is the normalized oxygen pressure and Y is the fractional oxygen saturation of the haemoglobin. Following the suggestion of Edelstein (1971), the normalized oxygen pressures were calculated by multiplying the observed oxygen pressures in kPa by the constant value of 17.5 kPa^{-1}, corresponding to the average oxygen binding constant for the isolated α and β chains as determined by Brunori, Noble, Antonini & Wyman (1966).

The dose-response curves shown in Figure 4 were fitted (Powell, 1970) using the equation $R = ax/(b+x)$ where a and b are fitted constants, R is the oxygen liberated at 2 kPa divided by the maximum possible oxygen release at the same pressure, and x is the concentration of drug.

Molecular modelling

The working model (Beddell *et al.*, 1973) was at first used as a basis for designing compounds. However, it became clear that a more accurate representation was needed of the DPG receptor site of human deoxyhaemoglobin. This was constructed (Norrington, 1974) at a scale of 2×10^8: 1 from atomic coordinate positions kindly supplied by Dr M.F. Perutz, and was used to make accurate measurements of the distances between atoms in the protein and interacting atoms in models of trial molecules.

Choice of compounds

The DPG receptor site of human deoxyhaemoglobin is an exceedingly basic region in the protein. Each β chain contributes its terminal amino group and three basic amino acid residues ($\text{His}^{\beta 2}$, $\text{Lys}^{\beta 82}$ and $\text{His}^{\beta 143}$), giving a total of eight basic nitrogen functions in the immediate site. Two more basic residues ($\text{Lys}^{\beta 144}$ and $\text{His}^{\beta 146}$) from each β chain are nearby. Arnone (1972)

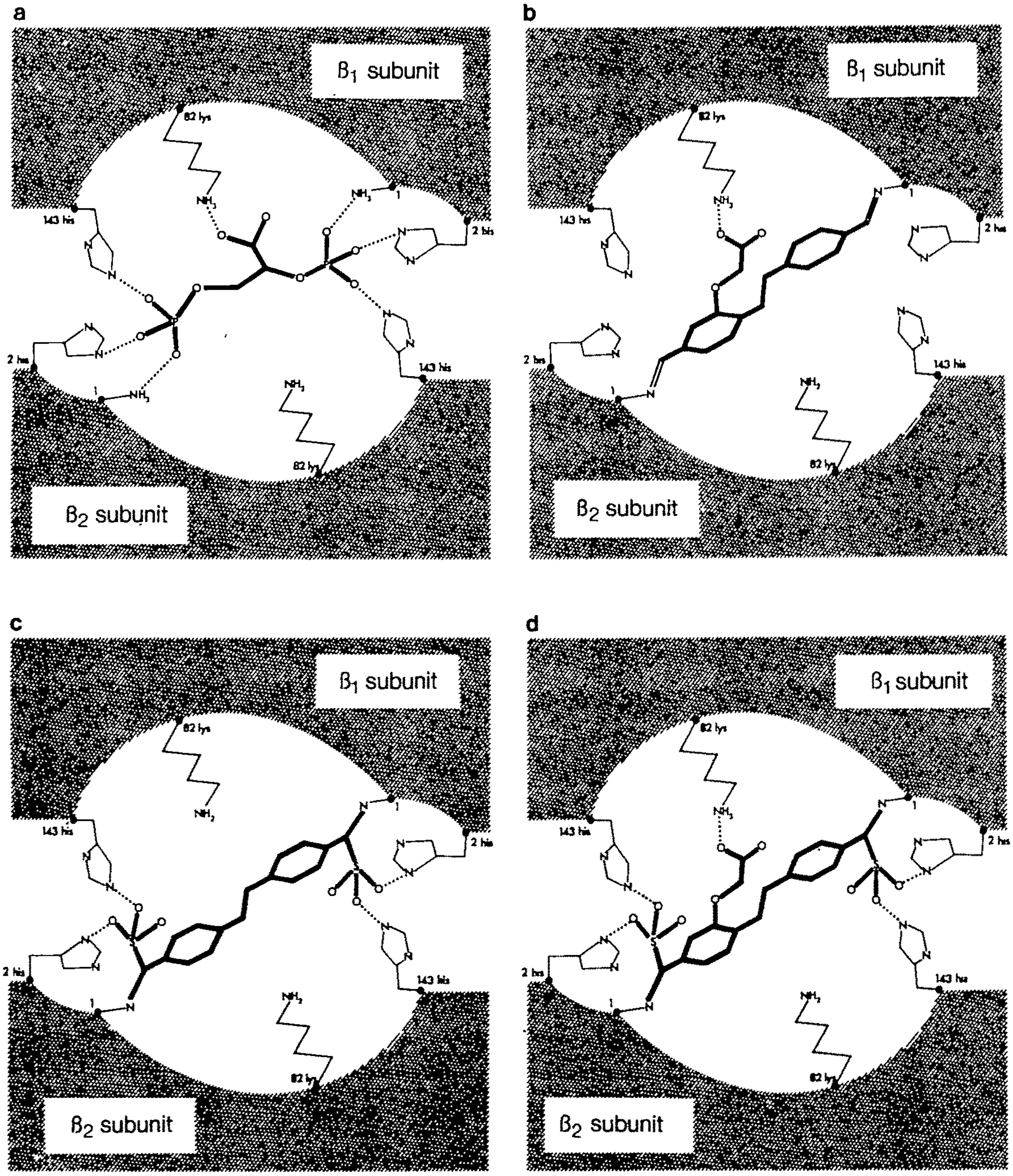
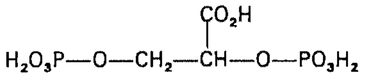


Figure 1 Schematic representations of the 2,3-diphosphoglycerate (DPG) receptor site: (a) with DPG bound as observed by Arnone (1972); (b) the postulated mode of binding of Compound I; (c) the postulated mode of binding of Compound II; (d) the postulated mode of binding of Compound III.

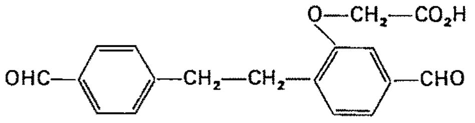
showed that each of six oxygen atoms in the phosphate groups of DPG is in a position to interact with one of the basic nitrogen functions of the receptor, while a seventh carboxyl oxygen might be interacting (Figure 1a). In attempting to design alternative types of compound to react with this region of the protein it was desirable to avoid highly ionized molecules like DPG itself, because their ionic character would inhibit passage across the red cell membrane. This property enables DPG to accumulate in the red cell after its metabolic formation, but would prevent exogenous compounds from reaching their intended intracellular site of action. We were also anxious, if possible, to design compounds of a completely different chemical type from DPG, and it was desirable to impose certain limitations on the conformational flexibility of the compounds. For these and other reasons attention was directed to the terminal amino groups of the β chains, and it was noted that the bibenzyl structure, in the conformation observed by Robertson (1934), would nearly span the distance between these amino groups in human deoxyhaemoglobin. The introduction of two aldehyde substituents to give bibenzyl-4,4′-dialdehyde provided reactive groups which were sterically matched to react with the terminal amino groups of the deoxy-protein by Schiff base formation. Moreover the aromatic rings of the bibenzyl structure imparted the intended degree of rigidity to bibenzyl-4,4′-dialdehyde, and model-building studies confirmed that it could not remain similarly bound when the protein was in the oxy conformation. It was therefore reasonable to hope that this compound would bias the haemoglobin equilibrium in favour of the deoxy form, and thereby promote the liberation of oxygen in a manner analogous to DPG itself.

High aqueous solubility had not been expected for bibenzyl-4,4′-dialdehyde, but it was actually found to be so insoluble in water that it could not be tested on haemoglobin solutions. It was therefore necessary to modify the structure in an attempt to increase solubility, and if possible provide more reactive groups to react with the protein. Compound I was therefore prepared, and this was sufficiently soluble for testing at concentrations up to 2.5 mmol dm^{-3}. It also seemed possible that the carboxylate group could interact (Figure 1b) with $\text{Lys}^{\beta 82}$, since it was in a favourable position to do so and Arnone (1972) had suggested that the carboxylate group of DPG might interact in this manner.

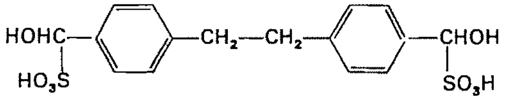


$$H_2O_3P{-}O{-}CH_2{-}\overset{\displaystyle CO_2H}{\overset{|}{C}H}{-}O{-}PO_3H_2$$

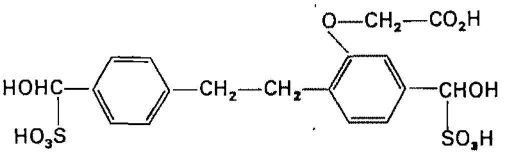
(DPG)



(I)



(II)



(III)

An alternative method of solubilizing bibenzyl-4,4′-dialdehyde was also investigated, by converting it to the bisulphite derivative (Compound II). This was soluble and could be tested at concentrations up to 5 mmol dm^{-3}, but it was necessary to add 5 mmol dm^{-3} sodium metabisulphite to the solution because some dialdehyde tended to precipitate unless free bisulphite ions were also present. Two possible modes of interaction between Compound II and the DPG receptor site may be considered. The bisulphite derivative might be in equilibrium with a sufficiently high concentration of the free dialdehyde for that to react by Schiff base formation with the terminal amino groups of the β chains as originally envisaged. However, it is well established (Lauer & Langkammerer, 1935; Shriner & Land, 1941; Caughlan & Tartar, 1941; Sheppard & Bourns, 1954) that the bisulphite derivatives of aldehydes are α-hydroxysulphonic acids, and Compound II might therefore interact with the terminal amino groups and

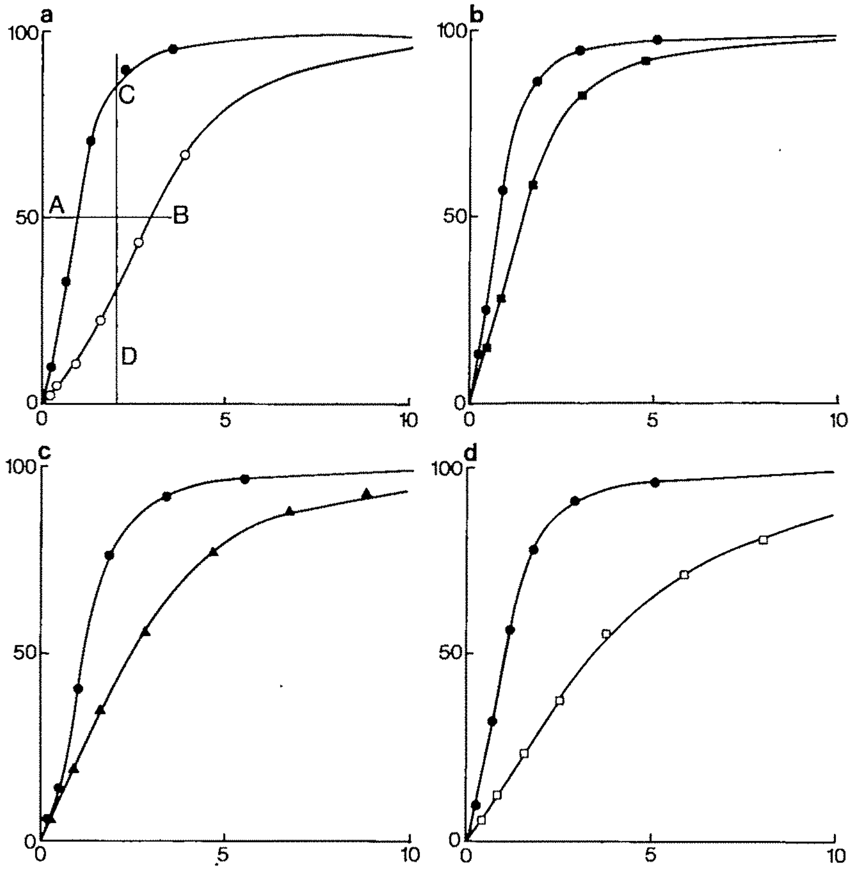


Figure 2 The oxygen dissociation curves of human haemoglobin in the presence and absence (●) of the compounds at a concentration of 2.5 mmol dm^{-1}. Abscissae, the gas pressure of oxygen in kPa. Ordinates, percent of oxyhaemoglobin. (a) Observations with 2,3-diphosphoglycerate (O); (b) observations with Compound I (■), note the diminished right shift and reduced oxygen liberation at constant pressure; (c) observations with Compound II (▲); (d) observations with Compound III (□).

some of the other basic residues of the DPG site, perhaps as illustrated schematically in Figure 1c. The bisulphite derivative (Compound III) of Compound I was also prepared, and since this has the extra carboxyl group available for interaction, we expected it to be the most potent of the compounds studied. Figure 1d illustrates one possible way in which it might interact.

Preparation of compounds

Bibenzyl-4,4′-dialdehyde was prepared according to the method of Reichstein & Oppenauer (1933). 4,4′-Diformyl-2-bibenzyl-oxyacetic acid (Compound I) was obtained by carboxymethylation of the corresponding phenol prepared by reduction of the stilbene obtained from the Wittig reaction (cf. Reimann, 1969) between triphenyl-(4-carbethoxybenzyl)-phosphonium bromide and 3-hydroxy-4-formyl benzoic acid. The bisulphite derivatives (Compounds II and III) were prepared by addition of an aqueous solution of sodium metabisulphite to an alcoholic solution of the dialdehyde.

Free 2,3-diphosphoglyceric acid was obtained by ion exchange on Amberlite IR-120(H) from the pentacyclohexylammonium salt (Calbiochem A Grade), and a 25 mmol dm^{-3} solution of the potassium salt was prepared by titration to pH 7.35 with potassium hydroxide solution. Aliquots were stored at −20°C.

Test solutions were prepared containing either 2,3-diphosphoglycerate or the appropriate test compound. Sodium metabisulphite 5 mmol dm^{-3} was also added to solutions of bisulphite derivatives in order to stabilize these derivatives. HEPES buffer (Good, Winget, Winter, Connolly, Izawa & Singh, 1966) at pH 7.35, haemoglobin solution, sodium chloride and water were added to give a final haemoglobin con-

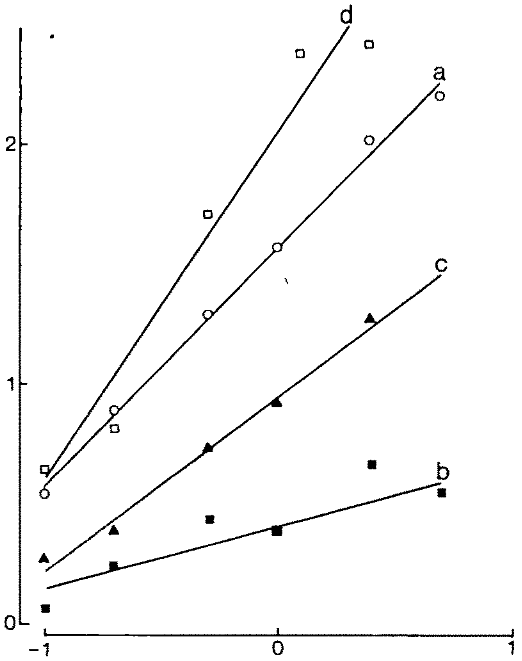


Figure 3 Logarithmic abscissae, concentration of compound in mmol dm^{-3}. Ordinates, right shift in the P_{50} value of the haemoglobin dissociation curves (kPa). (a) Observations with 2,3-diphosphoglycerate (○); (b) observations with Compound I (■); (c) observations with Compound II (▲); (d) observations with Compound III (□).

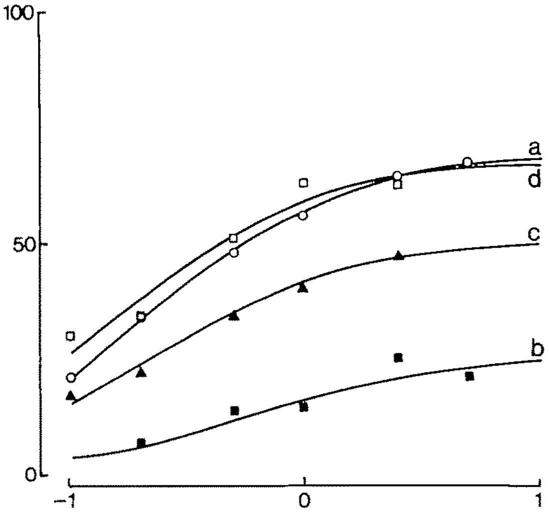


Figure 4 Logarithmic abscissae, concentration of compound in mmol dm^{-3}. Ordinates, oxygen liberated from the haemoglobin, at a constant pressure of 2 kPa, expressed as a percentage of the maximum amount of oxygen which could be liberated from the haemoglobin at that pressure. (a) Observations with 2,3-diphosphoglycerate (○); (b) observations with Compound I (■); (c) observations with Compound II (▲); (d) observations with Compound III (□).

centration of 30 µmol dm^{-3}, and an ionic strength contribution from test compound, sodium metabisulphite and sodium chloride of 35 mmol dm^{-3}.

Results

Control observations with DPG

Figure 2a shows oxygen dissociation curves for human haemoglobin in the absence and presence of 2.5 mmol dm^{-3} DPG. The shift to the right demonstrates the decreased oxygen affinity which is caused by this compound. It may be measured by the oxygen pressure at equilibrium with a mixture of 50% oxyhaemoglobin and 50% deoxyhaemoglobin (the P_{50} value). This is given by the intersection of line AB with the curves. Six different DPG concentrations have been studied, and Figure 3a shows the shift of P_{50} as a function of the logarithm of the concentration of DPG.

The observations may also be expressed as the amount of oxygen liberated from haemoglobin by different concentrations of DPG at a constant partial pressure of oxygen. Such an interpretation is more relevant to the physiological function of DPG, which is to control the amount of oxygen liberated from the blood at the partial pressure of the capillaries. The intersections of the curves with the line CD, which corresponds to an arbitrary pressure of 2 kPa, are plotted against the logarithm of the concentration of DPG in Figure 4 (line a). The distance CD represents the total amount of oxygen carried by the haemoglobin in the absence of DPG at a gas pressure of 2 kPa, and is thus the maximum response which could be measured by this test system under these physiological conditions. It represents 100% response in Figure 4, and the effect of DPG has been fitted to a sigmoid which provides an adequate fit to the observations.

Observations with compound I

Figure 2b shows the dissociation curve in the presence and absence of 2.5 mmol dm^{-3} of Compound I. This compound is less potent than DPG, as shown by the smaller shifts of the P_{50} plotted against log concentration in Figure 3 (line b). The amount of oxygen liberated at a constant pressure of 2 kPa is also much smaller (Figure 4, line b), and the sigmoid fitted to the observations shows an asymptotic maximum at only 26% response, which may be compared with the fitted asymptote of 70% for DPG. One may conclude that this compound does promote the liberation of oxygen as intended, but that it does so weakly. A lower potency was expected, however, since the compound was designed to interact with fewer groups in the receptor site than DPG itself (Figure 1b).

Observations with compound II

Sodium metabisulphite 5 mmol dm^{-3} was added to solutions containing Compound II, in order to prevent the free dialdehyde from precipitating. Control observations were therefore carried out to test whether this inorganic salt had any effect on the dissociation curve by itself. Although the addition of 5 mmol dm^{-3} sodium metabisulphite slightly modified the dissociation curve, the effect was always smaller than that caused by only 0.1 mmol dm^{-3} DPG.

Compound II was less potent than DPG but more potent than Compound I as judged by the right shift of the P_{50} value (Figure 3, line c), or the amount of oxygen liberated at 2 kPa oxygen pressure (Figure 4, line c). The greater potency would be expected if some of the oxygen atoms of the sulphonate groups were able to interact with the basic nitrogen atoms in the DPG site, and this type of interaction is shown schematically in Figure 1c.

Observations with Compound III

Sodium metabisulphite 5 mmol dm^{-3} was also added to solutions of Compound III. This compound had a substantial effect on the dissociation curves (Figure 2d), showing a comparable potency to DPG itself as measured both by the right shift (Figure 3, line d) and the oxygen liberated at 2 kPa pressure (Figure 4, line d). Once again this was to be expected if the compound interacted with several of the basic nitrogen atoms at the DPG receptor site, as shown schematically in Figure 1d.

Discussion

The present experiments were carried out in order to test the feasibility of designing drugs to interact with a receptor site of known molecular structure, and produce predefined biological effects. The work is open to many criticisms, and it is still incomplete. In particular, there is as yet no direct evidence on the mechanism by which the novel compounds influence the haemoglobin-oxygen dissociation curve, nor the site at which they interact with the protein. However, we believe that a study such as the present investigation should be carried out prospectively because it is very easy to justify the choice of compounds *post hoc propter hoc* once the mode of molecular interaction has been established. It is improbable that the predicted interactions as illustrated schematically in Figure 1 are correct in every detail. However, when the true mechanisms have been established it should be instructive to analyse the errors in the present predictions.

It has been common practice to design new drugs by modifying the chemical structure of a known substance which has the desired biological properties, and this procedure has imposed severe restraints on the choice. However, it is not necessary for the novel compounds to be related to the original substance when the structure of the receptor site is already known. In the present case the starting substance was a sugar phosphate, and we were rapidly and logically led to the consideration of aromatic dialdehydes from a study of the receptor site. In our opinion it is most unlikely that we would have made this transition from one chemical class to another, if we had applied the more conventional procedures. Designing new compounds by the method of receptor fit therefore seems to offer a substantial advantage in this respect.

Interaction with a biological receptor is not the only important factor in designing drugs. It might also be necessary to optimize uptake or distribution *in vivo*, and such aspects are often related to the physicochemical properties of compounds (Hansch & Fujita, 1964). In the present work, for example, the solubility of bibenzyl-4,4′-dialdehyde was not adequate and various modifications of this structure were therefore considered. The present approach again shows an advantage over traditional procedures in this respect, because it was possible to select compounds for synthesis which should not only have greater solubility, but should also interact more favourably with the receptor site.

In the present study the selected compounds produced the desired biological effect, and to this extent at least the approach has been successful. Moreover it was possible to design novel compounds which were not closely related to the natural starting substance DPG, and the first structure chosen could be modified logically in order to improve both its fit to the receptor and its physicochemical properties at the same time. This suggests that the method may prove to be both efficient and flexible. In fact it was not even necessary to postulate a unique mechanism of receptor interaction for the designed compounds, and we have not yet established whether they form Schiff bases, or interact without covalent bond formation, or even just rely on hydrogen bonding to the protein. It is now necessary to determine the mode of binding in order to optimize receptor fit, but the structure of native haemoglobin is already known and Fourier difference maps should identify the location of each compound unless it grossly distorts the crystal structure. The method of designing drugs to fit their receptors may therefore prove to be more economical in this respect than would be expected at first sight, and should be widely applicable because new protein structures are being published with increasing frequency. It would now seem appropriate for more of these structural studies to be specifically directed at enzymes which were selected in the first place as potential targets for therapy.

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NATURE OF HISTAMINE RECEPTORS IN THE EMETIC CHEMORECEPTOR TRIGGER ZONE

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1 The protective effects of the intracerebroventricular (i.c.v.) administration of the H_1-receptor antagonist, mepyramine and the H_2-receptor antagonists, burimamide and metiamide on centrally induced histamine-emesis were studied in unanaesthetized dogs.

2 The PD_{50} values of intraventricular mepyramine, burimamide and metiamide against the 100% emetic dose of histamine (3.0 mg i.c.v.) were found to be approximately 200 µg, 20 µg and 20 µg respectively.

3 Although burimamide (i.c.v. or i.v.) afforded protection against histamine-induced emesis, there was no protection against intravenous apomorphine- or oral copper sulphate-induced emesis.

4 The results suggest that both H_1- and H_2-histamine receptors in the emetic chemoreceptor trigger zone of the area postrema are concerned in histamine-induced emesis.

Introduction

The region of the area postrema in the medulla oblongata is highly vascular and there is an abundance of nerve terminals in the region (Cammermeyer, 1949; Brizzee & Neal, 1954). This is compatible with the concept of chemoreceptors for the emetic action of various chemicals (Borison & Wang, 1953). The area postrema is very rich in biogenic amines, including histamine (Adam, 1961) but their precise role in the emetic chemoreceptor trigger (ECT) zone is not known. However, intraventricular administration of histamine in dogs elicited emesis which was completely blocked after surgical ablation of the ECT zone (Bhárgava & Dixit, 1968). Histamine receptors for emesis were therefore proposed in the ECT zone but they were not classified. With the availability of specific H_1- and H_2-receptor antagonists, it is now possible to assess the receptor type involved in the central emetic action of histamine. The present work was designed to determine the protective effects of the H_1-receptor antagonist, mepyramine and the H_2-receptor antagonists, burimamide and metiamide on histamine-induced emesis.

Methods

The investigation was carried out in mongrel dogs weighing between 8 and 14 kg of either sex. Chronic implantation of stainless steel cannulae into either of the lateral cerebral ventricles was performed aseptically under pentobarbitone sodium anaesthesia (25 mg/kg, i.v.) according to the technique of Bhargava, Gupta & Chandra (1961). Streptomycin 0.25 g plus procaine penicillin 200,000 u, intramuscularly, was given post-operatively for 5 days. The correct placement of cannulae was ascertained by withdrawal of clear cerebrospinal fluid from the implanted cannulae, by a positive emetic response to an injection of apomorphine (2 µg) into the cannulated lateral ventricle 3 to 4 days after the implantation and finally on autopsy.

Experiments were designed to study the effect of mepyramine, burimamide and metiamide on the emetic response to parenterally and intracerebroventricularly (i.c.v.) administered histamine. For studying the emetic response, the dogs were fed 30 min before the injection and observed till they vomited, or for a period of 2 hours. The volume of drug solutions injected into the lateral cerebral ventricle never exceeded 0.5 ml. Tests were made at intervals of at least 3 days.

Drugs employed in the study were histamine dihydrochloride (Ward, Blenkinsop Co.), apomorphine hydrochloride (Mallinckrodt Chemicals, New York), copper sulphate, mepyramine maleate (May & Baker, Bombay), burimamide and metiamide (SK & F). The doses of histamine refer to the base, those of other drugs to their salts.

Results

The protective effects of mepyramine, burimamide and metiamide against the emesis induced by intracerebroventricular histamine are summarized in

Table 1. The doses of each histamine antagonist (i.c.v.) which protected 50% of the animals (PD_{50}) against the 100% emetic dose (3 mg) of histamine were determined. The PD_{50} of mepyramine (200 µg) was found to be approximately ten times that of burimamide and metiamide (20 µg).

It was found in a series of dogs, that the 100% emetic dose of histamine (i.c.v.) had to be increased four-fold to overcome the effect of the PD_{50} doses of burimamide and mepyramine given 30 min before histamine challenge.

The antiemetic activity of intravenous as well as intraventricular burimamide was tested against the emetic response induced by intravenous and intraventricular histamine and apomorphine and oral copper sulphate. The results (Table 2) show that whereas burimamide given either intravenously or intraventricularly was effective in protecting against intraventricular histamine-induced emesis, intraventricular burimamide offered only partial protection against intravenous histamine. Intravenous apomorphine (50 µg/kg) as well as oral copper sulphate (300 mg)-induced emesis was unaffected by intravenous or intraventricular burimamide treatment.

Discussion

Although it has been proposed that histamine receptors in the ECT zone of the area postrema of the dog may fully account for the emesis induced by intraventricular histamine (Bhargava & Dixit, 1968), the nature of these receptors was not known. The present study has clearly shown the importance of both H_1- and H_2-receptors in the emetic action of centrally administered histamine. Several investigators (Black, Duncan, Durant, Ganellin & Parson, 1972; Saxena, 1975) have reported roles for H_1- and H_2-receptors in the peripheral vasculature. Similarly in the brain, histamine-induced cyclic adenosine 3′,5′-

Table 1 Antiemetic action of mepyramine, burimamide and metiamide against 100% emetic dose (3.0 mg i.c.v.) of histamine in dogs

Antiemetic agent (µg, i.c.v.)		*No. of dogs that vomited*	*% Vomited*	*Approximate 50% protective dose (µg)*
Mepyramine	125	2/3	66	200
	250	1/3	33	
	500	1/5	20	
	1000	0/5	0	
Burimamide	10	2/3	66	20
	25	2/4	50	
	50	1/5	20	
	100	0/6	0	
Metiamide	10	3/4	75	20
	25	3/6	50	
	50	1/5	20	
	100	0/6	0	

Table 2 Antiemetic activity of burimamide

Burimamide pretreatment		*100% Emetic challenge*		*No. of dogs that vomited*	*% Protection*
Route	*Dose (µg/kg)*	*Route*	*Dose*		
	Nil	Histamine i.v.	1 mg/kg	7/7	0
i.v.	100	Histamine i.v.	1 mg/kg	1/5	80
i.v.	200	Histamine i.v.	1 mg/kg	0/5	100
i.c.v.	100 (total)	Histamine i.v.	1 mg/kg	2/4	50
i.v.	100	Histamine i.c.v.	3 mg (total)	0/4	100
i.v.	200	Apomorphine i.v.	50 µg/kg	5/5	0
i.c.v.	100 (total)	Apomorphine i.v.	50 µg/kg	3/3	0
i.v.	500	Copper sulphate orally	300 mg (total)	3/3	0
i.c.v.	100–5000 (total)	Copper sulphate orally	300 mg (total)	2/2	0

monophosphate activity (Schwartz, 1975) and temperature changes (Lomax, Green & Cox, 1975) are mediated through H_1- and H_2-receptors.

Our PD_{50} studies, against histamine-induced emesis, with the antagonists of H_1- and H_2-receptors revealed that the H_2 antagonists, burimamide and metiamide were approximately 10 times more potent than mepyramine which is a H_1 antagonist. However, since a four-fold increase in the 100% emetic dose of intraventricular histamine overcomes the effects of equipotent (PD_{50}) doses of both H_1- or H_2-receptor blocker, it is possible that both receptors have an equal significance in the emetic response to histamine.

The selective antiemetic action of burimamide is shown by its ineffectiveness against intravenous apomorphine- and oral copper sulphate-induced emesis. These results rule out a general non-specific depressant action of the H_2 antagonist on the emetic centre. Furthermore, the lack of antagonism of burimamide against intravenous apomorphine- or oral copper sulphate-induced emesis suggests that histamine receptors are not involved in all types of emesis. It is interesting to note the failure of burimamide (i.c.v) to offer full protection against intravenously induced histamine emesis. Although not studied in a quantitative fashion this could indicate that part of the emesis induced by parenterally administered histamine (Peng & Pi, 1967) is mediated through central pathways not involving histamine. Neither histamine nor burimamide is believed to cross the blood-brain barrier (Halpern, Neveu & Wilson, 1959; Cross, 1973). However, the results of the present study show that intravenous burimamide antagonizes intraventricular histamine and that intravenous histamine can be counteracted by intraventricular burimamide. Thus, these agents probably pass from the blood to the region of the area postrema in adequate concentrations to produce pharmacological responses. Possibly the area postrema, where the emetic ECT zone is situated, does not have a normal blood-brain barrier.

These experiments suggest that H_2-receptor antagonists may have clinical significance in conditions where histamine may play a role in emesis such as anaphylaxis, motion-sickness and pregnancy and in which H_1-receptor antagonists have some activity (Bhargava, 1974).

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COMPARISON OF THE ACTIONS OF ACEBUTOLOL, PRACTOLOL AND PROPRANOLOL ON CALCIUM TRANSPORT BY HEART MICROSOMES AND MITOCHONDRIA

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1 The effects of acebutolol, practolol and propranolol (0.5–3 mM) on calcium uptake, calcium binding and ATPase activities of the rabbit and rat heart microsomal and mitochondrial fractions were investigated.

2 Dose-response and time course experiments revealed that propranolol greatly inhibited microsomal and mitochondrial calcium uptake whereas both acebutolol and practolol showed slight depressant effects.

3 The ATPase activities of microsomal and mitochondrial fractions were decreased by acebutolol, practolol and propranolol; however, the latter agent was more effective than the other two.

4 The inhibitory effects of acebutolol, practolol and propranolol on mitochondria and microsomes were not antagonized by adrenaline.

5 Propranolol decreased calcium binding by the microsomal fraction only, whereas acebutolol and practolol had no effect on microsomal or mitochondrial calcium binding.

6 The sensitivity of the rabbit heart subcellular fractions to the β-adrenoceptor blocking drugs was similar to that of the rat heart; however, the calcium uptake and ATPase activities of microsomes were more sensitive to propranolol than mitochondria in both species.

7 Perfusion of rat hearts with 0.2–1 mM propranolol decreased contractile force, and microsomal and mitochondrial fractions obtained from these hearts accumulated less calcium in comparison to the control. On the other hand, acebutolol and practolol (0.2–1 nM) had no appreciable effects on contractile force or subcellular fractions under similar conditions.

8 The negative inotropic effect of propranolol may partly be due to its inhibitory actions on calcium transport by subcellular organelles of the myocardium; the depressant action of propranolol on calcium transport is unlikely to be due to its β-adrenoceptor blocking property.

Introduction

The effects of some well known β-adrenoceptor blocking agents on subcellular membranes of the myocardium have been reported by various investigators. For example, propranolol has been shown to inhibit calcium transport and adenosine 5′-triphosphate (ATP) hydrolysis by the fragments of sarcoplasmic reticulum (microsomal fraction) (Scales & McIntosh, 1968; Hess, Briggs, Shinebourne & Hamer, 1968; White & Shinebourne, 1969; Shinebourne, White & Hamer, 1969; Solaro, Gertz & Briggs, 1972; Katz, Repke, Tada & Corkedale, 1974). This inhibitory effect of propranolol was reversed by catecholamines (Hess *et al.*, 1968; White & Shinebourne, 1969) while other investigators (Katz *et al.*, 1974) failed to confirm this finding. Thus it is not clear whether or not the depressant effect of propranolol on microsomal calcium transport is due to its β-adrenoceptor blocking activity. Although propranolol has also been shown to inhibit calcium uptake by mitochondria (Noack & Greeff, 1970; 1971), the interaction of propranolol with catecholamines has not been studied by employing these membranes. Furthermore, the data available in the literature do not permit any conclusion to be made concerning the comparative effects of propranolol on mitochondrial and microsomal calcium transport.

Recently acebutolol and practolol have been claimed to exhibit greater cardio-selectivity than propranolol with respect to their β-adrenoceptor blocking activity (Dohadwalla, Freedberg & Vaughan Williams, 1969; Coleman & Leary, 1972; Basil, Jordan, Loveless & Maxwell, 1973; 1974). However, the actions of both acebutolol and practolol on calcium transporting properties of cardiac subcellular particles have not yet been described. In the present investigation, therefore, we have compared the effects of acebutolol, practolol and propranolol on calcium binding, calcium uptake and ATPase activities of the mitochondrial and microsomal fractions of the rat and rabbit hearts. The calcium transporting abilities of these subcellular fractions obtained from rat isolated hearts perfused with these β-adrenoceptor blocking agents were also examined to test if these subcellular effects of the drugs are associated with functional changes in the myocardium. It should be noted here that both sarcoplasmic reticulum and mitochondria are considered to regulate intracellular calcium and subsequently myocardial function (Harigaya & Schwartz, 1969; Dhalla, Sulakhe, Fedelesova & Yates, 1974), although their relative contribution during cardiac contraction and relaxation phases is far from clear.

Methods

Isolation of subcellular membrane fractions

Healthy albino rabbits and Sprague-Dawley rats were used. After killing the animals, the hearts were dissected out immediately, and rinsed thoroughly in a chilled 0.25 mM sucrose solution containing 1 mM disodium edetate (EDTA), at pH 7.0. The mitochondrial and heavy microsomal fractions were isolated by differential centrifugation according to the procedures described by Sulakhe & Dhalla (1971). In brief, the myocardium (ventricles) was homogenized in a medium containing 0.25 mM sucrose and 1 mM EDTA, pH 7.0; the mitochondrial fraction was obtained upon centrifugation at 10,000 *g* for 20 min whereas the heavy microsomal fraction was that pelleted upon spinning at 40,000 *g* for 40 minutes. Both mitochondrial and microsomal fractions were washed twice with homogenizing medium and 0.6 M KCl, pH 6.8 respectively and the final pellets of these subcellular membrane particles were suspended in 0.25 mM sucrose, pH 7.0. These membrane fractions were used immediately after isolation which was carried out at temperatures between 0° and 4°C.

Determination of calcium uptake and calcium binding

Both ATP-dependent Ca^{2+} uptake and Ca^{2+} binding by the isolated membrane fractions were determined by the Millipore filtration technique (Sulakhe & Dhalla, 1972). Calcium uptake by mitochondrial fraction was studied by incubating the membranes (0.1–0.3 mg protein) in a total volume of 1 ml reaction medium containing (mM): KCl 100, $MgCl_2$ 10, sodium succinate 5, KH_2PO_4 4, ATP 5, $^{45}CaCl_2$ 0.1, Tris-HCl 20, pH 7.0 at 37°C, whereas the microsomal Ca^{2+} uptake was studied by incubating the membrane fraction (0.02–0.05 mg protein) in 1 ml medium containing KCl 100, $MgCl_2$ 10, potassium oxalate 5, ATP 5, $^{45}CaCl_2$ 0.1, Tris-HCl 20, pH 7.0 at 37°C. ATP-dependent Ca^{2+} binding ability of the mitochondria or microsomes was studied by incubating the membranes (0.1–0.3 mg protein) in the same media as employed for calcium uptake experiments except that KH_2PO_4, succinate and potassium oxalate were omitted. The temperature of the reaction medium for calcium binding experiments was 25°C. The membrane fractions were pre-incubated for 2 to 3 min in the absence or presence of different drugs before starting the calcium accumulation reaction by the addition of $^{45}CaCl_2$. The reaction was terminated by Millipore filtration at various time intervals.

Drug solutions at desired concentrations were prepared fresh by dissolving the agents in deionized water and their pH was adjusted to 7.0.

Measurement of ATPase activities

Total ATPase activity of the mitochondrial and microsomal fractions was measured by incubation of the membrane fraction in the same reaction mixture as that used for the calcium binding experiments. The membrane fractions were pre-incubated for 3 min with or without drugs at 37°C before the reaction was started by adding ATP. The reaction was terminated by Millipore filtration at appropriate times. Inorganic phosphate in the protein-free filtrate was determined by the method described by Taussky & Shorr (1953) and the protein concentration was measured according to the method of Lowry, Rosebrough, Farr, & Randall (1951).

Perfusion of rat hearts

Perfusion of rat heart by the method of Langendorff is described elsewhere (Dhalla, Yates, Walz, McDonald & Olson, 1972). The control perfusion medium, Krebs-Henseliet solution containing 1.25 mM $CaCl_2$ was gassed with 95% O_2 and 5% CO_2, pH 7.2 at 37°C. The hearts were equilibrated by perfusing with the control medium for 10 min before starting perfusion with medium in the presence of drugs. The control hearts were perfused with Krebs-Henseliet solution only. The contractile force of the perfused hearts was monitored by employing a force-displacement transducer (FT.03) on a Grass polygraph (Dhalla *et al.*, 1972).

Results

Calcium binding and uptake by subcellular fractions

The rabbit and rat heart mitochondrial fractions in the presence of 4 mM inorganic phosphate (Pi)

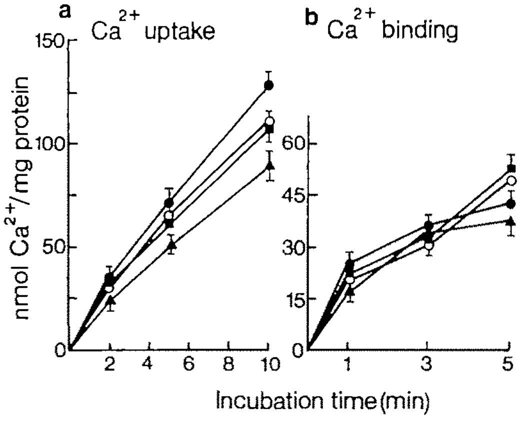

Figure 1 Effects of 3 mM acebutolol (○), 3 mM practolol (■) and 1 mM propranolol (▲) on (a) calcium uptake and (b) calcium binding activities of rat heart mitochondrial fraction at different intervals of incubation. (●) Control. Each value is a mean of 6 to 8 experiments. Vertical lines show s.e. mean.

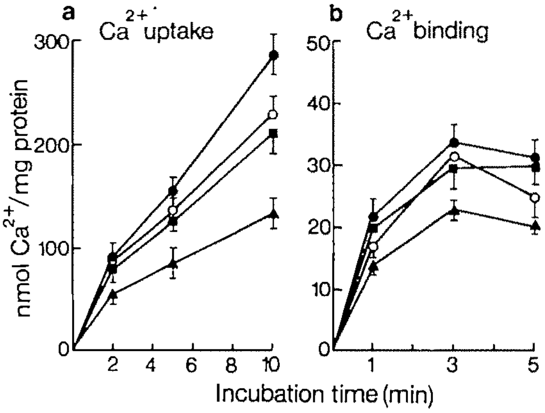

Figure 2 Effects of 3 mM acebutolol (○), 3 mM practolol (■) and 1 mM propranolol (▲) on (a) calcium uptake and (b) calcium binding activities of rat heart microsomal fraction at different intervals of incubation. ●, Control each value is a mean of 6 to 8 experiments. Vertical lines show s.e. mean.

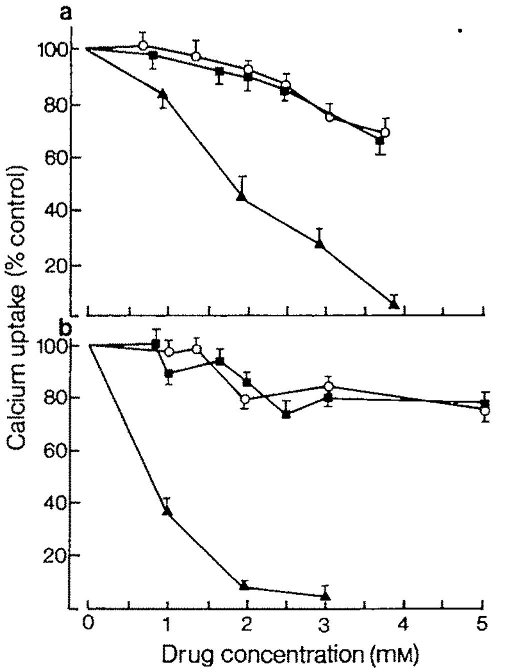

Figure 3 Effects of different concentrations of acebutolol (○), practolol (■) and propranolol (▲) on calcium uptake by (a) mitochondrial and (b) microsomal fractions of rabbit heart. The calcium uptake abilities of the subcellular fractions were determined for a period of 10 min and the results are expressed as % of the control values in the absence of drugs. Each value is a mean of 6 experiments. Vertical lines show s.e. mean.

(mitochondrial calcium uptake) accumulated 280 ± 18 and 132 ± 15 nmol Ca^{2+}/mg protein whereas the microsomal fractions in the presence of 5 mM potassium oxalate (microsomal calcium uptake) accumulated 610 ± 32 and 287 ± 27 nmol Ca^{2+}/mg protein in 10 min respectively. On the other hand, the rabbit and rat heart mitochondrial fractions in the absence of Pi (mitochondrial calcium binding) bound 65 ± 4 and 38 ± 3 nmol Ca^{2+}/mg protein whereas the microsomal fractions in the absence of oxalate (microsomal calcium binding) bound 44 ± 3 and 33 ± 2 nmol Ca^{2+}/mg protein in 5 min respectively. The calcium binding and calcium uptake by the mitochondrial fractions, unlike microsomal fractions, were decreased by 65 to 85% by 5 mM sodium azide, a well known inhibitor of mitochondrial calcium transport (Sulakhe & Dhalla, 1971). Electron miscroscopic and marker enzyme studies (Sulakhe & Dhalla, 1971) revealed minimal cross contamination in the fractions employed in this study. It should be noted that our values for the calcium binding and uptake abilities by the rat and rabbit heart subcellular fractions are within the accepted range reported by different investigators and it is understood that the terms 'calcium binding' and 'calcium uptake' imply an arbitrary meaning for calcium accumulation in the absence and presence of permeant ions respectively.

Time course of drug effects on calcium binding and uptake

In one series of experiments, the calcium binding and calcium uptake abilities of subcellular fractions were studied at different intervals of incubation with or without drugs. The results for rat heart are shown in Figures 1 and 2. Both acebutolol and practolol (3 mM) exerted a slight depressant action on mitochondrial and microsomal calcium uptake; however, this effect was significant ($P < 0.05$) only at 10 min of incubation with calcium (Figures 1 and 2). On the other hand, propranolol (1 mM) significantly decreased calcium uptake by mitochondrial and microsomal fractions at 2, 5 and 10 min of incubation. Calcium binding by mitochondria (Figure 1) was not significantly ($P > 0.05$) affected by acebutolol, practolol or propranolol whereas calcium binding by the rat heart microsomes (Figure 2) was significantly ($P < 0.05$) depressed by propranolol (1 mM) but not by acebutolol or practolol (3 mM). Similar results were obtained for the rabbit heart subcellular fractions. It should be pointed out that it is essential to adjust the pH of the drug solution to 7.0 before starting the incubation. For example, practolol, which is available as a free base, forms a highly alkaline solution and depressed mitochondrial and microsomal calcium uptake by about 70 to 82% at 3 mM concentration. This action of the alkaline solution of propranolol was found to be due to changes in the pH of the incubation medium rather than to the drug *per se*.

Dose-response of drugs on calcium uptake

The effects of different concentrations of acebutolol, practolol and propranolol were studied on the calcium uptake abilities of mitochondrial and microsomal fractions of rat and rabbit hearts. The results shown in Figure 3 and Table 1 indicate that both acebutolol and practolol at 2 to 3 mM concentrations decreased calcium uptake by both mitochondrial and microsomal fractions slightly but significantly ($P < 0.05$) whereas propranolol had a marked depressant action. The effectiveness of propranolol in decreasing calcium uptake by the subcellular fractions was qualitatively similar for both rat and rabbit hearts (Figure 3 and Table 1); however, mitochondrial calcium uptake was less sensitive to propranolol than microsomal calcium uptake in both species.

Effect of drugs on ATPase activity

In addition to accumulating calcium, both mitochondrial and microsomal fractions are known to hydrolyze ATP. The effects of drugs were therefore studied on ATPase activities of the subcellular fractions and the results for rabbit heart are given in Table 2. Both acebutolol and practolol (3 mM) decreased mitochondrial and microsomal ATPase activities significantly ($P < 0.05$) whereas a significant depressant effect of propranolol was apparent even at 1 mM. The mitochondrial ATPase was found to be less sensitive to propranolol than the microsomal ATPase of the rabbit heart. Similar results were also seen with rat heart subcellular fractions.

Table 1 Effects of different concentrations of acebutolol, practolol and propranolol on calcium uptake by rat heart mitochondrial and microsomal fractions

	*% of control Ca^{2+} uptake**	
Drugs (mM)	*Mitochondria*	*Microsomes*
Acebutolol		
0.5	102 ± 5	99 ± 5
1.0	97 ± 4	92 ± 6
2.0	92 ± 3**	86 ± 4**
3.0	78 ± 6**	76 ± 5**
Practolol		
0.5	98 ± 8	100 ± 12
1.0	96 ± 2	94 ± 10
2.0	90 ± 3**	86 ± 4**
3.0	77 ± 3**	75 ± 5**
Propranolol		
0.5	91 ± 11	60 ± 12**
1.0	72 ± 12**	37 ± 10**
2.0	43 ± 8**	12 ± 3**
3.0	24 ± 6**	9 ± 2**

* Each value is a mean ± s.e. of 5 experiments. Calcium uptake in the absence (control) and presence of drugs was determined for a period of 10 min and the results are expressed as % of the control value.

** Significant inhibition ($P < 0.05$).

Table 2 Effects of different concentrations of acebutolol, practolol and propranolol on ATPase activity of rabbit heart mitochondrial and microsomal fractions

	*ATPase activity** *(μmol Pi mg⁻¹ min⁻¹)*	
Drugs (mM)	*Mitochondria*	*Microsomes*
Control	1.54±0.10	1.60±0.12
Acebutolol		
1	1.45±0.16	1.33±0.08
2	1.49±0.13	1.29±0.11
3	1.14±0.09**	1.08±0.10**
Practolol		
1	1.55±0.15	1.35±0.12
2	1.39±0.08	1.39±0.12
3	1.11±0.11**	1.16±0.08**
Propranolol		
1	1.22±0.13**	1.04±0.11**
2	0.95±0.10**	0.76±0.09**
3	0.74±0.09**	0.55±0.08**

* Each value is a mean±s.e. of 5 to 6 experiments. The total ATPase activity was determined for a period of 5 minutes.

** Significant inhibition ($P < 0.05$).

Table 3 Contractile force and calcium uptake by mitochondrial and microsomal fractions obtained from rat hearts perfused for 10 min in the absence (control) or presence of different concentrations of acebutolol, practolol and propranolol

	Calcium uptake (%)*		*Contractile force (%)*
Drugs (mM)	*Mitochondria*	*Microsomes*	
Acebutolol			
0.2	104±5	110±7	102±5
0.5	95±3	115±7	95±6
1.0	91±5	93±8	91±4
Practolol			
0.2	95±3	92±4	107±2
0.5	96±4	99±7	109±3
1.0	94±4	99±5	102±4
Propranolol			
0.2	91±6	89±2**	87±3**
0.5	85±3**	70±8**	32±5**
1.0	67±4**	56±6**	0.8±0.1**

* Each value is a mean±s.e. of 4 experiments. Calcium uptake was studied for a period of 5 min and the results are expressed as % of the control.

** Statistically significant ($P < 0.05$).

Interaction of adrenaline with drugs on subcellular fractions

Adrenaline in concentrations from 10 to 150 μM was found to have no effect upon the abilities of mitochondrial and microsomal fractions to accumulate calcium as well as to hydrolyze ATP; however, higher concentrations of adrenaline were slightly inhibitory. The depressant actions of 1 mM propranolol, 3 mM acebutolol and 3 mM practolol on mitochondrial and microsomal calcium uptake (as shown in Figures 1 and 2) and ATPase activities (as shown in Table 2) were not antagonized by the addition of 10 to 150 μM adrenaline in the incubation medium.

Effect of drug perfusion on contractile force and subcellular fractions of rat heart

In order to test whether or not acebutolol, practolol and propranolol affect mitochondrial and microsomal calcium uptake *in vivo*, rat hearts were perfused with different concentrations of these agents for 10 minutes. The contractile force of these hearts was monitored, the subcellular fractions isolated and their abilities to accumulate calcium were examined. The results of this series of experiments are shown in Table 3. It was found that both acebutolol and practolol at 0.2 to 1 mM had no significant effect on the contractile force nor were there any appreciable changes in calcium transporting abilities of mitochondrial and microsomal fractions obtained from these drug-treated hearts. On the other hand, the hearts perfused with 0.2 to 1 mM propranolol showed a progressive depression in contractile force and yielded subcellular fractions which accumulated less calcium in comparison to those from the control hearts. The depression in calcium accumulating ability of the microsomal fraction from the propranolol-treated hearts was greater than that by the mitochondrial fraction. It should be pointed out that the concentrations of acebutolol, practolol and propranolol employed in this study completely prevented the positive inotropic effect of 0.5–1 μg adrenaline, which produced 65 to 90% increase in contractile force of the isolated perfused heart of the rat.

Discussion

In this study we have shown that propranolol inhibited heart microsomal calcium uptake in rat and rabbits. This finding is in agreement with observations reported by various investigators using dog and calf cardiac muscle preparations (Scales & McIntosh, 1968; Hess *et al.*, 1968; Shinebourne *et al.*, 1969; Katz *et al.*, 1974). Furthermore, our results concerning the depression of mitochondrial calcium uptake by propranolol with rabbit and rat hearts as measured by the Millipore filtration technique also confirm the findings of Noack & Greeff (1971) who used atomic absorption spectrophotometric measurements on the rat heart. In addition, we have shown that two other β-adrenoceptor blocking agents, acebutolol and practolol, decreased calcium uptake by mitochondrial and microsomal fractions; however, in contrast to propranolol, the depressant effect of these agents was seen in higher concentrations. Since acebutolol and practolol are more cardioselective β-adrenoceptor blocking agents than propranolol (Dohadwalla *et al.*, 1969; Coleman & Leary, 1972; Basil *et al.*, 1973; 1974), it is unlikely that the depressant effect of these agents is related to their β-adrenoceptor blockade. This view is supported by our observation that the mitochondrial and microsomal effects of acebutolol, practolol and propranolol were not antagonized by a β-adrenoceptor agonist, adrenaline. On the other hand, the inhibitory action of propranolol is considered to be some non-specific effect on the physico-chemistry of microsomal membranes (Lameijer & Van Zwieten, 1974) and has been shown to be antagonized by sucrose (Solaro *et al.*, 1972). The depression in microsomal calcium transport by propranolol has also been suggested to be due to inhibition of the ATPase activity (Scales & McIntosh, 1968) and of the γ-AT^{32}P reaction (Pang & Briggs, 1973). It is possible that the observed effects of acebutolol and practolol on microsomal and mitochondrial membranes are elicited through mechanisms similar to those for propranolol.

Calcium uptake by mitochondrial and microsomal fractions obtained from hearts perfused with different concentrations of propranolol was found to be less than the control value; mitochondrial calcium uptake decreased to a lesser degree in comparison to the microsomal calcium uptake. Furthermore, microsomal calcium uptake was depressed to a greater extent than mitochondrial calcium uptake when the subcellular fractions were incubated with propranolol. This difference in the sensitivity of mitochondrial and microsomal calcium uptake to propranolol cannot be attributed to the difference in experimental conditions employed in this study because propranolol was found to decrease microsomal calcium binding, unlike mitochondrial calcium binding, under similar conditions of incubation. Furthermore, mitochondrial ATPase was less sensitive to propranolol than microsomal ATPase. These differences may be due to different chemical compositions of mitochondrial and microsomal membranes with respect to their lipid and protein contents. It can also be seen from the data reported by Nayler (1966) that propranolol was more effective in inhibiting microsomal than mitochondrial lipid-facilitated transport of calcium.

Although rat heart subcellular fractions transported less calcium in comparison to the rabbit heart fractions, the degree of depression in calcium uptake due to propranolol in both species was qualitatively similar. It is noteworthy that we have been able to show decreased ability for Ca^{2+} uptake in subcellular fractions obtained from hearts depressed to varying degrees upon perfusion with different concentrations of propranolol. On the other hand, acebutolol and practolol, which unlike propranolol, did not depress myocardial contractility significantly in the concentrations employed in this study, had no appreciable action on the calcium transporting abilities of mitochondrial and microsomal fractions obtained from these drug-treated hearts. Thus it is likely that changes in calcium transport properties of heart mitochondria and sarcoplasmic reticulum due to propranolol may represent the subcellular basis of its negative inotropic action. Since mitochondria were less sensitive to propranolol than microsomes, it appears that sarcoplasmic reticulum may play a major role in this mechanism. It should also be noted here that the present study does not rule out the possibility of propranolol acting on the sarcolemma which is also known to transport calcium like other membrane systems of the myocardium.

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INDIRECT AND DIRECT INHIBITION OF DOPAMINE β-HYDROXYLASE BY AMPHETAMINE IN STORAGE VESICLES AND SYNAPTOSOMES

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1 To elucidate the mechanism by which amphetamine produces a functional inhibition of dopamine β-hydroxylase (DBH), the actions of amphetamine on amine uptake and β-hydroxylation were examined in isolated adrenal storage vesicles and in whole brain synaptosomes of the rat.

2 Amphetamine produces a competitive inhibition of catecholamine incorporation into adrenal vesicles with a Ki of 279 μM; this action of the drug accounted for slightly less than half of the total inhibition of β-hydroxylation in the vesicles, indicating that the interference by amphetamine of access of substrate to the enzyme compartment plays an important role in functional inhibition of DBH. The remainder of the effect in vesicles probably represents direct inhibition of the enzyme.

3 In synaptosomes, similar actions on uptake of substrate and on functional enzyme activity were noted, indicating that amphetamine-induced inhibition of the neuronal amine pump can also affect DBH activity if the substrate is supplied exogenously.

4 In addition to the effects of amphetamine *in vitro*, chronic administration of amphetamine to rats produced an increase in total activity of adrenal DBH.

5 The net effect of amphetamine on DBH activity thus represents the summation of direct inhibition of the enzyme, indirect inhibition via reduced access of substrate and enhancement of activity via trans-synaptic induction.

Introduction

Among the many actions of amphetamine on sympathetically innervated tissues is the ability of the drug to produce a functional inhibition of dopamine β-hydroxylase (DBH) both *in vitro* (Goldstein & Contrera, 1962) as well as *in vivo* (Stolk, 1975). Because DBH is contained almost entirely within the adrenergic storage vesicles (Stjärne & Lishajko, 1967), Viveros, Arqueros, Connett & Kirshner, 1969), it is difficult to identify the specific site for this functional inhibition. Amphetamine undoubtedly causes direct inhibition of DBH (Goldstein & Contrera, 1962), but in systems in which the vesicles remain intact, blockade of the vesicular uptake mechanism by amphetamine may produce indirect inhibition by preventing access of dopamine to the enzyme (Slotkin & Kirshner, 1971; Slotkin, Ferris & Kirshner, 1971). Additionally, if β-hydroxylation is measured in synaptosomal preparations or *in vivo* by administration of a radioactive substrate, then the precursor amine must first be taken up into the intraneuronal extravesicular space, and amphetamine also inhibits this process (Coyle & Snyder, 1969; Horn, Coyle & Snyder, 1971). Thus, in a number of studies, the specific actions of amphetamine on DBH activity *in vivo* have been difficult to interpret (Baird & Lewis, 1964; Taylor & Snyder, 1971; Stolk, 1975).

In the present experiments, the actions of amphetamine in lysed or intact adrenal storage vesicles and in synaptosomes are compared in order to determine which of the three factors (direct inhibition of the enzyme, blockade of synaptosomal uptake, blockade of vesicular uptake) play major roles in functional inhibition of DBH activity.

Methods

Experiments in vivo

Male Sprague-Dawley rats (200–250 g, Zivic-Miller) were given (+)-amphetamine sulphate (5 mg/kg, i.p.) every 12 h for 10 days, while controls received 0.9% w/v NaCl solution (saline). Twelve hours after the last injection, the animals were decapitated, and the adrenal glands were homogenized (glass-to-glass) in 2.5 ml of 0.3 M sucrose, buffered at pH 7.4 with 0.025 M Tris-sulphate, containing 10^{-5} M iproniazid; 0.1 ml of the homogenate was taken for catecholamine analysis (Merrills, 1963) and 0.5 ml of the homogenate, added to 0.5 ml of water, was used for duplicate assays of DBH activity (Friedman & Kaufman, 1965); *p*-hydroxymercuribenzoate (optimal concentration: 0.5 mM) was added to the DBH incubation mixture to inactivate endogenous inhibitors, and [^{3}H]-tyramine (10^{-5} M) was used as the substrate.

The remaining homogenate was centrifuged at 800 *g* for 10 min, and two 0.5 ml portions were used as a source of storage vesicles for uptake determinations (*vide infra*). The rest of the 800 *g* supernatant was centrifuged at 26,000 *g* for 10 min and duplicate 0.1 ml portions withdrawn for assays of tyrosine hydroxylase activity (Waymire, Bjur & Weiner, 1971) using [^{14}C]-tyrosine (10^{-4} M) as the substrate.

Uptake of [^{3}H]-adrenaline into isolated adrenal storage vesicles from control and amphetamine-treated rats was evaluated as described previously (Slotkin & Kirshner, 1973a); 0.5 ml of the 800 *g* supernatant was added to 0.5 ml of sucrose-Tris containing ATP-Mg^{2+} (5 µmol), [^{3}H]-adrenaline (3 µCi) and unlabelled adrenaline (0.1 µmol; to obviate differences in extravesicular catecholamine concentrations among samples). The samples were incubated for 30 min at 30°C while duplicate tubes were kept on ice to serve as blanks. The labelled vesicles were centrifuged 10 min at 26,000 *g*, washed twice, lysed and analyzed for radioactivity and catecholamines. Under these conditions, labelling occurs solely in storage vesicles despite the presence of contaminant organelles (Slotkin & Kirshner, 1973b). The results were expressed as the temperature-dependent component of uptake per gland.

Experiments in vitro

Adrenal glands from untreated rats were homogenized in sucrose-Tris-iproniazid and portions were diluted with water to lyse the vesicles as described above; DBH activity in the lysed preparations was measured in the presence and absence of 1 mM amphetamine using [^{3}H]-tyramine (10^{-4} M) as substrate. The remaining homogenate, containing intact vesicles, was incubated and washed as described for the [^{3}H]-adrenaline uptake studies, except that the incubation mixture contained ATP-Mg^{2+} (5 µmol), fumarate (1 µmol), ascorbate (1 µmol), [^{3}H]-tyramine (5 µCi) and unlabelled tyramine (0.1 µmol), with and without amphetamine. The washed, labelled vesicles were analyzed both for tyramine uptake and for their content of the β-hydroxylated product, octopamine (Slotkin & Green, 1975).

To evaluate the effects of amphetamine on [^{3}H]-adrenaline uptake *in vitro*, the vesicle-containing 800 *g* supernatant from adrenal glands of untreated rats was incubated in the presence and absence of amphetamine (0.5 mM) as described, except that the amount of unlabelled adrenaline added to the medium ranged from 0.01–0.04 µmol; labelled vesicles were then washed and analyzed for catecholamines and radioactivity. Since the vesicular uptake system appears to follow saturation kinetics (Slotkin, 1975), results are reported as double reciprocal plots, enabling calculations of the Km for adrenaline and Ki for amphetamine.

The effects of amphetamine on catecholamine efflux from isolated adrenal storage vesicles were evaluated as described previously (Green & Slotkin, 1973). Vesicle-containing 800 *g* supernatants from untreated rats were pooled and incubated at 30°C for periods ranging from 5 to 60 min in the presence and absence of amphetamine (1 mM). Efflux was stopped by the addition of ice-cold sucrose-Tris, and samples were centrifuged at 26,000 *g* for 10 min and supernatants and pellets analyzed for catecholamines. The percentage of amine remaining in the vesicles was calculated as described previously (Slotkin *et al.*, 1971).

To determine the actions of amphetamine in intact synaptosomes, whole brains from untreated rats were homogenized in 9 vol of sucrose-Tris-iproniazid, centrifuged at 1000 *g* for 10 min, and the supernatant centrifuged at 11,000 *g* for 20 minutes. The crude synaptosomal pellet was resuspended in the same volume of modified Krebs-Henseleit medium containing iproniazid (1.25×10^{-6} M) and ascorbate (2×10^{-6}M) and recentrifuged. The washed pellet was resuspended by gentle homogenization (Teflon to glass) in half the original volume of Krebs-Henseleit medium and 0.5 ml portions were added to 0.5 ml of medium containing 5 µCi of [^{3}H]-tyramine (0.75 nanomole). Samples were incubated for 5 min at 37°C in the presence and absence of varying concentrations of amphetamine, while duplicate tubes were kept on ice to serve as blanks. Tyramine uptake was stopped by the addition of 2 ml of ice-cold Krebs-Henseleit medium and the labelled crude synaptosomes were sedimented at 11,000 *g*, washed and recentrifuged twice and analyzed for radioactivity and octopamine synthesis as described for adrenal vesicles. Centrifugation of the labelled crude fraction in sucrose density gradients (Bosmann & Hemsworth, 1970) indicated that the [^{3}H]-tyramine was indeed taken up primarily into synaptosomes.

Statistics

Data are reported as mean ± s.e. mean and levels of significance are calculated by Student's *t* test (Wine, 1964). Uptake kinetics are presented as double-reciprocal plots (Lineweaver & Burk, 1934); the slopes, intercepts and standard errors are calculated by the method of least squares (Wine, 1964).

Materials

(±)-Adrenaline-[7-^{3}H], tyramine-[G-^{3}H] and (–)-tyrosine-[1-^{14}C] were purchased from New England Nuclear Corp. Iproniazid phosphate, tyramine hydrochloride and (+)-amphetamine sulphate were obtained from Sigma Chemical Corp., and (–)-adrenaline bitartrate from Winthrop Laboratories.

Results

The chronic administration of amphetamine (5 mg/kg) produced little or no alteration in either adrenal catecholamine concentrations or in the ability of isolated adrenal storage vesicles to incorporate [^{3}H]-adrenaline (Table 1). However, the activities of both tyrosine hydroxylase and dopamine β-hydroxylase (DBH) were elevated substantially (130% and 40% above controls, respectively).

In contrast, direct addition of amphetamine to the medium containing the adrenal vesicles produced effective inhibition of uptake (Figure 1). The effect appeared to be competitive, with a Ki for amphetamine about 10-fold higher than the Km for adrenaline. Since vesicular uptake represents the summation of two processes, inward transport minus efflux (Slotkin, 1975), it was important to establish whether the inhibitory effect of amphetamine represented a reduction in transport or an acceleration of efflux. Amphetamine *in vitro* had no effect on the rate of loss of catecholamines from the vesicles (Figure 2), indicating that the blockade of uptake probably results from an effect on inward transport.

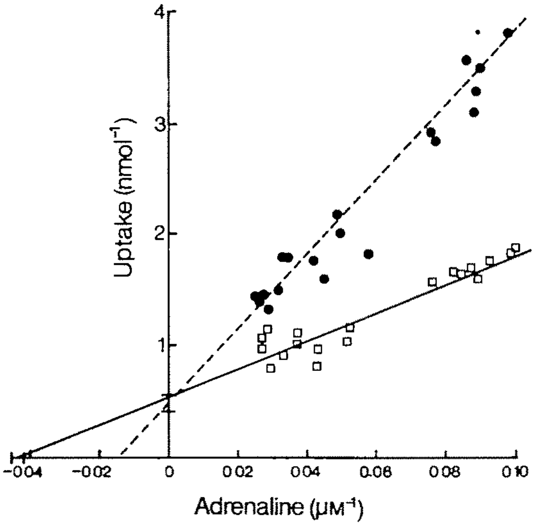

Figure 1 Lineweaver-Burk plot of adrenaline uptake into isolated rat adrenal medullary storage vesicles in the presence (●) and absence (□) of amphetamine (0.5 mM). Intercepts on the ordinate scale are significantly different ($P<0.001$), while intercepts on the abscissa are not. For adrenaline uptake without amphetamine, Km = 23 ± 1 μM and maximal uptake = 1.80 ± 0.08 nmol per gland; with amphetamine, Km = 64 ± 6 μM and maximal uptake = 2.02 ± 0.18 nmol per gland. Ki calculated for amphetamine = 279 ± 28 μM. Lines are drawn by least squares analysis.

Schneider (1972) has reported that amphetamine releases catecholamines from bovine isolated adrenal vesicles; the release is of a lytic nature, requiring the presence of salts (Ferris, Viveros & Kirshner, 1970). In the present study efflux was measured in sucrose, which leads to release via leakage of amines across intact vesicle membranes (Slotkin *et al.*, 1971) rather than lysis, and amphetamine did not alter this efflux.

Table 1 Effects of chronic amphetamine administration (5 mg/kg i.p., every 12 h for 10 days) on the rat adrenal medulla

Determination	*Control*	*Amphetamine*
Catecholamines (μg/gland)	8.91 ± 1.16	9.68 ± 0.78
[^{3}H]-Adrenaline uptake (nmol/30 min per gland)	2.15 ± 0.16	2.13 ± 0.21
Tyrosine hydroxylase (nmol $^{14}CO_2$ formed/h per gland)	14.9 ± 0.7	33.9 ± 2.8*
Dopamine β-hydroxylase (nmol [^{3}H]-octopamine formed/h per gland)	1.73 ± 0.08	2.47 ± 0.32*

Activities were measured *in vitro* after administration of amphetamine or saline *in vivo*. Each group consisted of six rats.

* $P<0.001$ vs. control

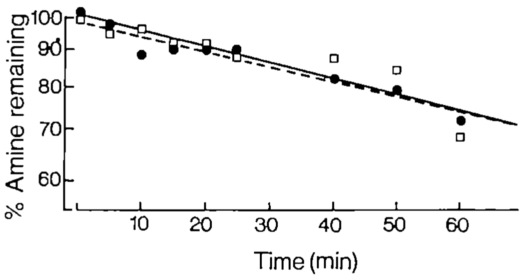

Figure 2 Efflux of endogenous catecholamines from isolated adrenal medullary vesicles in the presence (●) and absence (□) of 1 mM amphetamine, represented as percentage of amine remaining in the vesicles. Lines are drawn by least squares analysis; ordinate scale is logarithmic.

The ability or inability of amphetamine to cause accelerated loss of catecholamines thus probably depends upon the medium used.

Since many other amines besides adrenaline can utilize the vesicular transport system (Slotkin & Kirshner, 1971), it seemed likely that uptake-related effects of amphetamine on DBH could be evaluated by use of a DBH substrate which relies on uptake to gain access to the enzyme. One such amine is tyramine (Slotkin & Kirshner, 1971; Slotkin, 1975; Slotkin & Green, 1975). When amphetamine was added to a preparation of intact adrenal storage vesicles, the uptake of [^{3}H]-tyramine was reduced by 20% (Table 2). However, the net inhibition of β-hydroxylation was much larger (50%). Lysis of the vesicles prior to incubation produced a marked enhancement of [^{3}H]-octopamine synthesis compared

Table 2 Effects of amphetamine *in vitro* on tyramine uptake and octopamine synthesis in intact adrenomedullary vesicles, and on dopamine β-hydroxylase activity in lysed vesicles

Determination	*Control*	*Amphetamine (1 mM)*	*% inhibition*
Intact Vesicles			
[^{3}H]-Tyramine uptake (nmol/30 min per gland)	0.714±0.014	0.567±0.008*	21±1
[^{3}H]-Octopamine synthesis (nmol/30 min per gland)	0.316±0.006	0.159±0.002*	50±1
Lysed Vesicles			
[^{3}H]-Octopamine synthesis (nmol/h per gland)	12.6±0.2	6.07±0.10 *	52±1

Intact vesicle preparations were incubated 30 min with [^{3}H]-tyramine (0.1 mM) and values are reported for that period; lysed vesicles were incubated for 60 min with [^{3}H]-tyramine (0.1 mM). Seven determinations were done for each point.

* $P < 0.001$ vs. control

Table 3 Effects of amphetamine *in vitro* on tyramine uptake and octopamine synthesis in crude whole brain synaptosomes

	Amphetamine concentration		
Determination	*0*	*10^{-6} M*	*10^{-5} M*
[^{3}H]-Tyramine uptake (nmol/5 min per g of brain)	0.137±0.004	0.112±0.001†	0.084±0.003†
% inhibition	—	18±1	39±2
[^{3}H]-Octopamine synthesis (nmol/5 min per g of brain)	0.024±0.001	0.020±0.001*	0.013±0.0004†
% inhibition	—	17±4	46±2

Seven determinations were done for each point. [^{3}H]-Tyramine concentration in the medium was 7.5×10^{-7} M

* $P < 0.02$ vs. control.

† $P < 0.001$.

to the intact preparation; amphetamine produced slightly greater than 50% inhibition in the lysed vesicles.

Since tyramine also has an affinity for uptake into adrenergic neurones which is comparable to that of the natural catecholamine substrates (Burgen & Iversen, 1965), this compound was used also to determine the relative contribution of uptake inhibition by amphetamine to functional inhibition of DBH in crude synaptosomes prepared from whole brain homogenates. In the absence of amphetamine, approximately one-fifth of the [^{3}H]-tyramine taken up was converted to [^{3}H]-octopamine (Table 3). With increasing amphetamine concentrations, there was a progressive decline in both uptake and β-hydroxylation.

Discussion

The enclosure of dopamine β-hydroxylase (DBH) within a membrane-bound particle, the catecholamine storage vesicle, makes it possible to study the indirect inhibition of the enzyme, *i.e.* a reduction in activity by preventing access of substrate to the site of the enzyme. In situations where activity is measured in intact tissues or intact organelles, the uptake of substrate can be rate-limiting (Laduron, 1975); under these circumstances lysis of the vesicles should produce a large increase in activity, as illustrated in Table 2. It was therefore important to assess the degree to which amphetamine could inhibit uptake of substrate and whether the resulting indirect inhibition of DBH could account for a substantial proportion of the net effect of the drug. In isolated adrenal medullary vesicles incubated with amphetamine, there was a competitive inhibition of the catecholamine uptake system; the Ki for amphetamine was approximately the same as that obtained previously for other phenethylamine derivatives (Slotkin, Anderson, Seidler & Lau, 1975). The lack of effect of amphetamine on vesicular catecholamine efflux and the competitive nature of inhibition indicate that the site of action is probably the ATP-Mg^{2+}-stimulated transport system in the storage vesicle membrane (Slotkin, 1973, 1975).

Despite the ability of amphetamine to inhibit uptake of substrate into the vesicles, not all of the functional inhibition of DBH could be accounted for on the basis of reduced access; while vesicular uptake of tyramine was reduced by 20% by amphetamine, conversion to octopamine fell by 50%. These data suggest that even in intact organelles a substantial proportion of DBH inhibition by amphetamine results from a direct effect on the enzyme (Goldstein & Contrera, 1962; Stolk, 1975).

In contrast to the effects of amphetamine *in vitro*, administration of the drug to rats did not affect subsequently determined adrenal vesicular uptake of ^{3}H-adrenaline and actually increased adrenal DBH activity. The differences result from the reversible nature of amphetamine's effects in these systems (Stolk, 1975); because uptake and DBH activity were measured in adrenal homogenates, the inhibition by amphetamine was reversed during tissue preparation as the drug was washed out. On the other hand, functional inhibition of DBH by amphetamine has been observed readily after administration of substrate *in vivo* (Stolk, 1975). The increase in adrenal DBH and tyrosine hydroxylase activities after chronic administration probably represents trans-synaptic induction resulting from amphetamine-induced stimulation of the sympatho-adrenal axis (Mandell & Morgan, 1970; Koda & Gibb, 1973).

In addition to inhibiting amine uptake into storage vesicles, amphetamine blocks amine transport across the neuronal membrane (Coyle & Snyder, 1969; Horn *et al.*, 1971). Thus, in an intact organelle (synaptosome), the same indirect effect of amphetamine should be observed. In the current study, concentrations of amphetamine which inhibited tyramine uptake by 40% produced equivalent inhibition of β-hydroxylation, indicating that blockade of transport contributes substantially to inhibition of DBH. The amphetamine-induced reduction in uptake of DBH substrates from extracellular to intracellular sites is of questionable significance *in vivo* because under normal circumstances, dopamine is synthesized intracellularly and thus must be translocated only from the cytoplasm to the vesicle; however, in situations in which DBH activity is measured by administration of an exogenous substrate, inhibition of neuronal uptake by amphetamine can be a major factor in the net effect.

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EFFECT OF EXPERIMENTAL DIABETES, FOOD DEPRIVATION AND GENETIC OBESITY ON THE SENSITIVITY OF PITHED RATS TO AUTONOMIC AGENTS

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1 The sensitivities of alloxan and streptozotocin diabetic and hereditary obese pithed rats to acetylcholine, isoprenaline and noradrenaline were compared to those of controls.

2 Blood pressure and heart rate recordings made before dosing was started showed the streptozotocin-treated animals to have a significantly reduced heart rate and increased pulse pressure as compared with controls.

3 Both diabetic groups were found to have reduced sensitivities to the pressor effect of noradrenaline, the depressor effect of acetylcholine, the positive chronotropic and inotropic effect of isoprenaline and the reduction in diastolic pressure induced by isoprenaline. The reduction in sensitivity was generally much greater in the streptozotocin diabetic animals.

4 The genetically obese rats were found to have similar sensitivities to all three agents as did their non-obese litter mates.

5 When either diabetic group was deprived of food for 24 h preceding the tests the sensitivities were found to be raised significantly towards normal in almost all cases.

6 The results are contrasted with previous *in vitro* results and possible causative metabolic factors discussed. It is suggested that sensitivity changes are unevenly distributed within the cardiovascular system.

Introduction

Neurological and cardiovascular malfunctions are accepted as being much more common among diabetics than among the general population (Ellenberg, 1973; Wheeler & Watkins, 1973; Riff & Riff, 1974). Studies using experimentally diabetic animals have indicated changed sensitivities to autonomic agents, e.g. hyperresponsiveness to noradrenaline has been demonstrated in perfused rat hindquarters from alloxan diabetic animals (Brody & Dixon, 1964) and in isolated aortic strips from alloxan diabetic rabbits (Cseuz, Wenger, Kunos & Szentivany, 1973). However, a study using both alloxan and streptozotocin diabetic anaesthetized rats revealed no change in sensitivity to noradrenaline (Christlieb, 1974). This contrast may indicate that noradrenaline sensitivities vary in different parts of the cardiovascular system. Alternatively variations in the diabetogen used, its dose and the time allowed for the development of the diabetes may have resulted in animals with different patterns of metabolic and perhaps of consequent cardiovascular malfunctions being studied. Streptozotocin and alloxan have been reported to result in different forms of diabetes with high serum free fatty acid and ketone levels in alloxan-treated and more normal levels in streptozotocin-treated animals (Mansford & Opie, 1968). However, the pattern of metabolic changes depends also on the dose of diabetogen and the time allowed for its effects to develop (Schein, Alberti & Williamson, 1971; Bavli & Gordon, 1971).

A comparative study of the cardiovascular sensitivities to autonomic agents of streptozotocin and alloxan diabetic rats both fed and deprived of food and of genetically obese rats was carried out in order to try to link any changes in sensitivities with the diabetic state. The doses of the two diabetogens used and the periods allowed for development of diabetes had previously been found to result in similar levels of hyperglycaemia.

Methods

Noradrenaline, isoprenaline and acetylcholine sensitivities

All rats were pithed under ether and artificially respired, blood pressures recorded via carotid cannulae and drugs injected via a femoral vein. Heart rate was determined from the blood pressure recordings. Prior to dosing, a 0.1 ml sample of blood was withdrawn from each animal via the venous cannula for blood glucose determination. Responses to noradrenaline (100 ng, 300 ng, 1 μg and 3 μg/kg), isoprenaline (10 ng, 30 ng, 100 ng and 300 ng/kg) and acetylcholine (100 ng, 300 ng, 1 μg and 3 μg/kg) were recorded, time being allowed for recovery between each dose.

Alloxan and streptozotocin diabetic rats

Male C.F.E. rats (250–350 g) received 1 ml/kg of a freshly prepared solution of alloxan (50 mg/ml saline), streptozotocin (60 mg/ml pH 4.5 citrate buffer) or saline (0.9% w/v NaCl solution) alone via a tail vein. The last group served as controls. All rats were then allowed free access to food and water for one week in the case of the alloxan-treated and their controls and two weeks in the case of the streptozotocin-treated and their controls. Groups of alloxan and streptozotocin diabetic and control rats were then denied food for a further 20–28 h before test; these groups are referred to as 'starved' rats. All groups were allowed water *ad lib.*

Obese rats

Zucker obese rats were used at 2–3 months of age at which time their metabolic abnormalities should be present (Bray & York, 1971). At test their non-obese litter mates served as controls.

Blood glucose and free fatty acid determinations

Blood sugar was determined in the 0.1 ml blood samples withdrawn from the test animals by a micro-

Table 1 Blood glucose and free fatty acid determinations

Group	n	*Blood glucose (mg/100 ml)*	n	*Blood free fatty acids (nM)*
Controls fed	20	131.8 ± 8.0	6	0.1315 ± 0.329
Controls starved	6	73.9 ± 10.1**	5	0.1632 ± 0.0146
†ADR fed	16	553.7 ± 48.6***	9	0.1613 ± 0.0338
†ADR starved	18	214.1 ± 37.7***	6	0.1073 ± 0.0108
‡SDR fed	11	463.4 ± 42.2***	6	0.1206 ± 0.0149
‡SDR starved	11	183.0 ± 40.1***	6	0.1346 ± 0.0361
Zucker obese	6	206.0 ± 21.4		—
§L.M.	8	186.3 ± 29.6		—

†‡ Alloxan diabetic (ADR) and streptozotocin diabetic (SDR) rats respectively. § Non-obese litter mates of the Zucker obese rats.

* Significant differences between fed diabetic and fed controls and between starved and equivalent fed groups are indicated by asterisks: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 2 Mean blood pressure, heart rate and pulse pressure measurements of pithed rat groups before drug infusion

Group	n	⁺ *Mean blood pressure (mmHg)*	*Heart rate*	*Pulse pressure (mmHg)*
Controls fed	20	58.1 ± 2.1	271.4 ± 11.7	25.5 ± 1.6
Controls starved	15	59.8 ± 3.1	265.2 ± 14.9	23.7 ± 1.6
†ADR fed	12	52.9 ± 4.5	260.6 ± 20.1	25.8 ± 2.8
†ADR starved	10	55.1 ± 4.2	238.0 ± 13.6	20.5 ± 1.7
‡SDR fed	13	55.7 ± 2.6	197.2 ± 19.9***	44.5 ± 3.0***
‡SDR starved	18	52.7 ± 3.9	220.5 ± 20.1	31.1 ± 3.8
Zucker obese	9	62.8 ± 3.7	253.7 ± 22.2	29.6 ± 2.5
§LM	8	57.4 ± 2.4	238.6 ± 35.6	33.3 ± 3.7

⁺ Mean blood pressure is taken as diastolic + $\frac{1}{3}$ pulse pressure.

†‡ Alloxan diabetic (ADR) and streptozotocin diabetic (SDR) rats respectively. § Non-obese litter mates of the Zucker obese rats.

* Significant differences between fed diabetic and fed controls and between starved and equivalent fed groups are indicated by asterisks: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

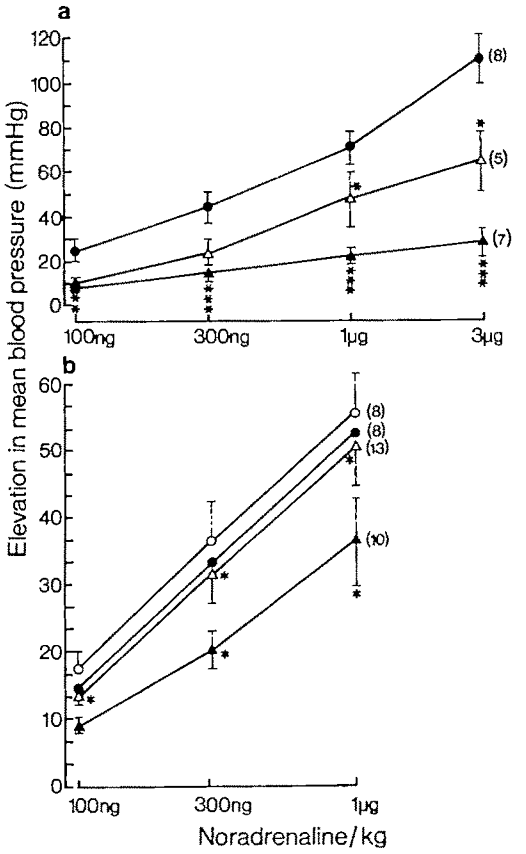

Figure 1 The effects of diabetes and starvation on the pressor response to noradrenaline. Each point represents the mean of *n* observations indicated in parentheses. Vertical lines show s.e. mean. (●) Fed controls; (○) starved controls; (▲) fed diabetic; (△) starved diabetic rats. (a) Effect of streptozotocin diabetes and starvation; (b) effect of alloxan diabetes and starvation. Significant differences between fed diabetic and fed controls and between starved and fed diabetic are indicated by: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

colourimetric copper reduction method (Varley, 1963). Free fatty acids were determined by a modification of the method of Itaya & Ui (1965), in 0.2 ml plasma samples from rats which had received the same treatment as the test animals. Determination of plasma free fatty acid levels in the obese rats was not carried out since these and other metabolic abnormalities are already well documented (Bray & York, 1971).

Statistical significance was assessed by Student's *t* test, *P* values of 0.05 or less being taken to indicate significance.

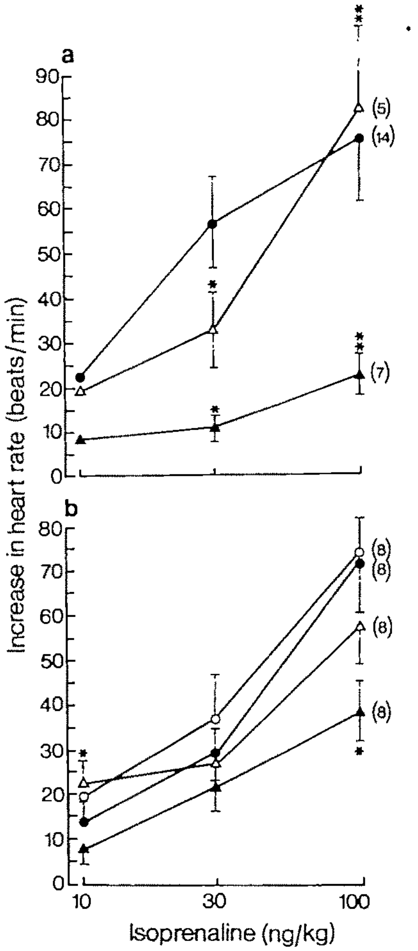

Figure 2 The effects of diabetes and starvation on the heart rate response to isoprenaline. Each point represents the mean of *n* observations indicated in parentheses. Vertical lines show s.e. mean. (●) Fed controls; (○) starved controls; (▲) fed diabetic; (△) starved diabetic rats. (a) Effect of streptozotocin diabetes and starvation; (b) effect of alloxan diabetes and starvation. Significant differences between fed diabetic and fed controls and between starved and fed diabetic are indicated by: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Results

Table 1 shows blood glucose and free fatty acid levels in control, diabetic and obese rats.

Streptozotocin (60 mg/kg i.v.) and alloxan (50 mg/kg i.v.) both produced marked hyperglycaemia at the time of test but neither had a significant effect on blood free fatty acid levels. Starving for 20–28 h significantly reduced blood glucose in controls and

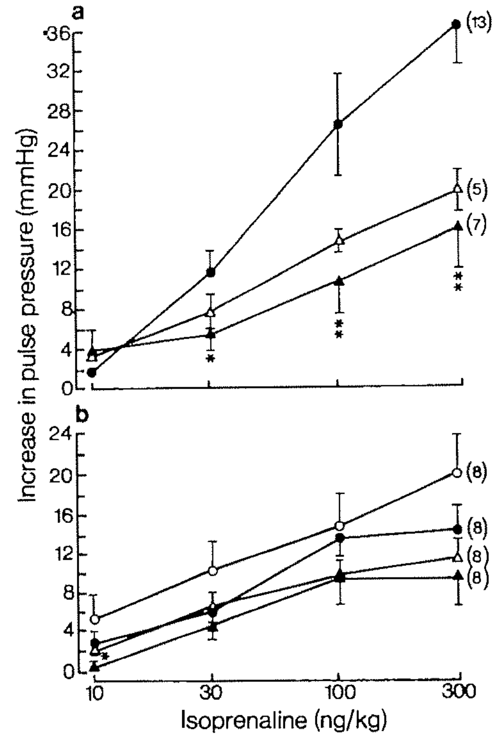

Figure 3 The effects of diabetes and starvation on the pulse pressure response to isoprenaline. Each point represents the mean of n observations indicated in parentheses. Vertical lines show s.e. mean. (●) Fed controls; (○) starved controls; (▲) fed diabetic; (△) starved diabetic. (a) Effect of streptozotocin diabetes and starvation; (b) effect of alloxan diabetes and starvation. Significant differences between fed diabetic and fed controls and between starved and fed diabetic are indicated by: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

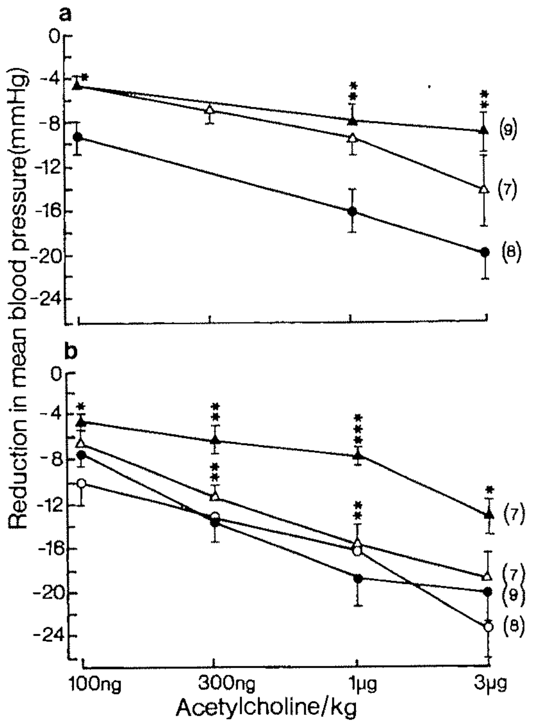

Figure 4 The effect of diabetes and starvation on the depressor response to acetylcholine. Each point represents the mean of n observations indicated in parentheses. Vertical lines show s.e. mean. (●) Fed controls; (○) starved controls; (▲) fed diabetic; (△) starved diabetic rats. (a) Effect of streptozotocin diabetes and starvation; (b) effect of alloxan diabetes and starvation. Significant differences between fed diabetic and fed controls and between starved and fed diabetic are indicated by: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

both diabetic groups but did not significantly change free fatty acid levels (Table 1).

There were no significant differences between any of the test groups in mean blood pressure. The fed streptozotocin diabetic group had a significantly reduced heart rate and increased pulse pressure compared to the fed controls. These values in the starved streptozotocin diabetic group were not significantly different from those of the fed controls (Table 2). Noradrenaline pressor responses (Figure 1), heart rate increases due to isoprenaline (Figure 2) and depressor responses to acetylcholine (Figure 4) were all significantly reduced at least at one dose level in both diabetic fed groups compared to their fed controls. Pulse pressure responses were also significantly reduced in the fed streptozotocin diabetic group (Figure 3a). Sensitivities to the pressor effect of noradrenaline (Figure 1) and the cardioaccelerator effect of isoprenaline (Figure 2) were raised significantly towards normal by starvation. Sensitivity to the depressor effect of acetylcholine was also significantly restored in the alloxan diabetic group (Figure 4b). The sensitivities of the obese rats to the agents used were not found to differ significantly from those of their controls. In the interest of simplicity therefore, results are not included.

Discussion

Sensitivity to all the three autonomic agents used was found to be reduced in both fed diabetic groups. The reduction in noradrenaline sensitivity contrasts with some previous work showing increased sensitivity in rat hindquarter vasculature (Brody & Dixon, 1964) and isolated aortic strip of the rabbit (Cseuz *et al.*, 1973). The possibility that differences were due to different diabetic models being studied must be considered. The animals used in the present experiments may be regarded as relatively mildly diabetic since they did not have the raised free fatty acid levels which characterize the more severe diabetic state. Brody & Dixon used much higher doses of alloxan (150 mg/kg) but they used the subcutaneous route of administration which, due to the short half life of alloxan *in vivo* (Leech & Baily, 1945; Patterson, Lazarow & Levey, 1949) produces a very variable degree of diabetes as indicated by the proportions of their animals which either died or failed to develop the disease. Since the more severely affected were probably among those that died, and those relatively unaffected were rejected from their test group, it is reasonable to assume that metabolically, their animals were similar to those used in the present study. It is therefore postulated that parts of the cardiovascular system other than those of the hindquarters become less sensitive to noradrenaline resulting in an overall decrease in pressor response. The positive chronotropic responses to isoprenaline were reduced in both fed diabetic groups (Figure 2) and this probably contributed to the reduced noradrenaline pressor effect. Since the cardiac response contributes only a small part to the pressor effect of noradrenaline its reduction cannot entirely account for the large decrease in the latter seen particularly in the streptozotocin-treated group. It, therefore, appears that overall vasoconstriction was also reduced. Hyperglucagonaemia has been found to occur in all forms of endogenous hyperglycaemia where it has been measured and may even be necessary for its development (Unger & Ora, 1975). The effect of glucagon on various vascular beds has been investigated. It has been found to reduce resistance (Bashour, Geumei, Nagrawi & Downey, 1973) and reduce the constrictor effects of noradrenaline, angiotensin and vasopressin (Richardson & Withrington, 1975) in the dog hepatic vasculature possibly by increasing extracellular potassium concentration (Hulstaert, Beijer, Brouwer, Teunissen & Charbon, 1974). Recent findings suggest a similar action in the rat mesenteric arteries but no such effect even at high concentrations was seen in the rat hindlimb vessels (Mishra, Sharma & Kishore, 1975). Hyperglucagonaemia may, therefore, contribute to the decreased noradrenaline pressor responses seen in the fed diabetic groups and account for the contrast between these results and those of the perfused rat hindquarter preparation. However, unless glucagon acts via an intermediate which is reduced by starvation, the return of sensitivity towards normal after starvation casts doubt on this hypothesis since hyperglucagonaemia along with hypoinsulinaemia and elevated blood free fatty acid and ketone levels are abnormalities of diabetes which are, if anything, made worse by starvation.

The most obvious metabolic abnormality of diabetes which is returned towards normal by starvation, hyperglycaemia, must therefore be considered as a causative factor in the reduced noradrenaline sensitivity of the fed diabetic groups. A reduction in constrictor responses of perfused rat mesenteries to both sympathetic nerve stimulation and exogenous noradrenaline when high glucose perfusate was used has been reported (Malik & McGiff, 1974). The reduction in response to exogenous noradrenaline by glucose concentration increases similar to those occuring *in vivo* in the present experiments however was not great and could only partly account for the large decrease in sensitivity, unless other large vascular beds were much more severely affected.

The reduction in depressor response to acetylcholine in both fed diabetic groups (Figure 4) coupled with the finding that the acetylcholine content of vagal nerve endings is reduced in the diabetic rat (Kuntscherova & Vlk, 1970) indicate impaired vagal function. This may be analagous to the impaired vagal function reported in some diabetic patients (Wheeler & Watkins, 1973). The return of sensitivity towards normal after starvation suggests hyperglycaemia as a causative factor.

The lack of significant changes in the cardiovascular responses of obese rats to the agents used compared to their non-obese litter mates indicates that the metabolic derangements of the obese rats, e.g. hyperlipaemia, hypercholestrolaemia and hyperinsulinaemia (for review see Bray & York, 1971) have no effect on such responses. For several reasons, however, this conclusion must be treated with caution: firstly the fact that these animals consist of about 50% fat may have resulted in a proportionally smaller blood volume because of the usually poorer blood supply to fat compared to most other tissues. This would have caused equivalent doses/kg to result in higher blood levels so that the lack of apparent change in sensitivity may have indicated lower actual sensitivity. Secondly, changes in cardiovascular sensitivity in one direction due to alteration in blood composition may have been masked by opposing changes due to metabolic or other abnormalities in the other direction. The net result could therefore be no overall change.

In conclusion, sensitivities to the three agents used were reduced in the fed diabetic but not in the obese rats and sensitivity was raised towards normal in the former by starvation. One of the causes of the reduced noradrenaline sensitivity hypothesized, namely hyperglucagonaemia, suggests a redistribution of blood supply away from the limbs in favour of the viscera.

Further investigations are at present being carried out to determine if this is the case. If so it may have a bearing on the vascular complications of the human diabetic which typically involve reduced peripheral blood supply.

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SELECTIVITY OF β-ADRENOCEPTOR AGONISTS AND ANTAGONISTS ON BRONCHIAL, SKELETAL, VASCULAR AND CARDIAC MUSCLE IN THE ANAESTHETIZED CAT

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1 The potencies of fifteen β-adrenoceptor agonists of widely differing chemical structures were compared with that of (−)-isoprenaline on bronchial muscle, soleus muscle, blood pressure and heart rate in the anaesthetized cat. The β-adrenoceptor antagonist potencies of propranolol and practolol were determined against (−)-isoprenaline in the same model.

2 (−)-Isoprenaline was the most potent agonist and its action was essentially unselective. Thus, on all four parameters the minimal effective dose was 0.003–0.01 µg/kg and maximal or near maximal responses were produced by 0.3–1 µg/kg. Trimetoquinol was also an essentially unselective agonist.

3 For thirteen of the remaining fourteen agonists, potency was similar on bronchial muscle, soleus muscle and blood pressure but significantly lower on heart rate.

4 The remaining agonist — AH 7616 (4-hydroxy-α^1-[[(1-methyl-3,3-diphenyl-propyl)amino]-methyl]-m-xylene-α^1,α^3-diol, acetate) – was also significantly less potent on heart rate than on the other parameters; in addition, it was clearly less potent on soleus muscle and blood pressure than on bronchial muscle when 5-hydroxytryptamine (5-HT) was used to induce bronchospasm. However, when acetylcholine was used instead of 5-HT the potency of AH 7616 on bronchial muscle, soleus muscle and blood pressure was very similar. AH 7616 may therefore possess a specific 5-HT antagonist action in addition to its β-adrenoceptor agonist action.

5 The fifteen test agonists were longer acting than (−)-isoprenaline and this was particularly true of trimetoquinol and soterenol.

6 The β-adrenoceptor antagonist potency of propranolol was almost identical on bronchial muscle, soleus muscle and blood pressure and very slightly lower on the heart. Practolol was 10–12 times more potent on the heart than on bronchial muscle, soleus muscle and blood pressure.

7 These findings suggest that it may not be possible to separate the bronchodilating and tremor-enhancing properties of β-adrenoceptor agonists. The results with agonists and antagonists are in accord with Lands' dual β-adrenoceptor sub-classification.

Introduction

β_2-Adrenoceptor agonists such as salbutamol (Cullum, Farmer, Jack & Levy, 1969) and terbutaline (Bergmann, Persson & Wetterlin, 1969) can produce effective bronchodilatation in man with relatively little accompanying cardiac stimulation (Legge, Gaddie & Palmer, 1971), but enhancement of physiological tremor has been noted as a side-effect, particularly after oral administration (Freedman, 1971; Legge *et al.*, 1971; Minette, 1971; Epstein, Barnard & Zsotér, 1973).

The enhancement of physiological tremor is thought to be mediated directly through β-adrenoceptors located in skeletal muscle (Marsden, Foley, Owen & McAllister, 1967; Marsden & Meadows, 1968); thus, β-adrenoceptor agonists decrease the degree of fusion and tension developed during submaximal tetanic contractions of the slow contracting motor units in human muscle (Marsden & Meadows, 1970). The same effects are produced in slow contracting skeletal muscle from animals, for example cat soleus muscle (Bowman & Zaimis, 1958). Bowman & Nott (1970) suggested therefore that the cat soleus would provide a useful model for forecasting tremor-enhancing potential in man. Using this preparation the skeletal muscle β-adrenoceptor has been classified as a β_2-type, like that in bronchial muscle (Bowman & Nott, 1970). From studies with a limited number of β-adrenoceptor agonists and antagonists it was concluded that differentiation of the β_2-adrenoceptors in these two tissues might not be possible (Bowman & Nott, 1970; Bowman & Rodger, 1972).

The present paper describes the results of a wide-ranging study of this problem. The effects of 16 β-adrenoceptor agonists and of two β-adrenoceptor antagonists on bronchial muscle, soleus muscle, blood pressure and heart rate have been evaluated in the anaesthetized cat. The results provide further information on two inter-related questions: first, the possibility of separating the tremor-enhancing and bronchodilating actions of β-adrenoceptor agonists; and second, the validity of the dual β-adrenoceptor classification of Lands and co-workers (Lands, Arnold, McAuliff, Luduena & Buzzo, 1967).

Methods

Fasted adult cats of either sex (2.1–3.3 kg body weight) were anaesthetized with α-chloralose (80 mg/kg i.v.) after induction with a halothane (3%), nitrous oxide (3 l/min), oxygen (2 l/min) mixture. Sodium pentobarbitone (3–6 mg/kg i.v.) was given as necessary to suppress spontaneous respiration. Bilateral vagotomy was carried out in all animals to minimize the effects of autonomic reflexes on bronchodilator and positive chronotropic responses to β-adrenoceptor agonists (Boissier, Advenier, Giudicelli & Viars, 1971; Vaughan Williams, Bagwell & Singh, 1973). Rectal temperature was monitored and maintained at 37 ± 1°C by means of a heated table (Palmer, London). All drugs were injected through a cannulated jugular vein. All parameters were recorded on a Devices (type M–4) pen recorder.

Tracheal pressure

Cats were artificially respired with room air by positive pressure ventilation with a Palmer Ideal pump. The stroke volume was 13 ml/kg body weight and the pump rate 28 strokes/minute. Intra-tracheal pressure (mmHg; 1 mmHg = 1.333 mbar) was measured from a side-arm of the tracheal cannula by the method of Dixon & Brodie (1903), modified by the use of an Ether (type UP1, ± 10″ WG) pressure transducer. In each cat the dose of 5-hydroxytryptamine (5-HT) required to produce an increase in resting intra-tracheal pressure of approximately 50% was determined; this varied between 2 and 20 µg/kg. This dose of 5-HT was injected every 10 min until constant bronchoconstrictor responses were obtained, and thereafter at 10 min intervals throughout the experiment. The lungs were inflated by occluding the pump outlet for three expirations following the peak of the bronchoconstrictor response to 5-HT in order to prevent atelectasis.

Soleus muscle

The left soleus muscle was prepared essentially as described by Bowman & Nott (1970). The sciatic nerve was stimulated through shielded bipolar platinum electrodes with rectangular pulses of 50 µs duration at twice the voltage required to elicit a maximal twitch (normally 10–25 V) delivered from a Farnell physiological stimulator (Farnell Instruments, Wetherby) *via* a radio-frequency isolation unit (type PI/U). Submaximal tetanic contractions (6–10 Hz) were elicited for 1 s every 10 s and isometric muscle tension recorded using an Ether (type UFI, 32 oz) strain gauge attached to the tendon of the soleus muscle. Skin flaps were raised to form a pool containing a mixture of equal amounts of warm liquid paraffin BP and 0.9% w/v NaCl solution (saline) maintained at 37 ± 1°C by means of a heating lamp and thermocouple. The strain gauge output was displayed on a Telequipment (type S51-A) oscilloscope at high gain so that resting tension could be accurately monitored. Resting tension was adjusted to give an 'optimal evoked twitch tension' (Bowman & Nott, 1970) at the start of each experiment and was maintained constant thereafter. This procedure is designed to ensure that contractions are comparable from experiment to experiment (Buller, Eccles & Eccles, 1960). The resting tension required varied between 40 and 110 g in individual experiments.

Cardiovascular system

Arterial blood pressure was monitored from a common carotid artery and recorded by means of a Consolidated Electrodynamics pressure transducer (type 4-327-L221). Heart rate was recorded using a Devices instantaneous ratemeter (type 2750) triggered by the pulse pressure.

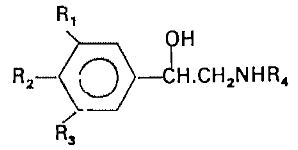
Experimental procedure

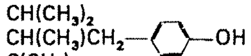
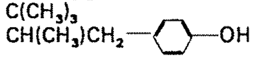
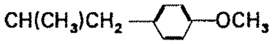
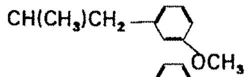
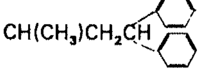
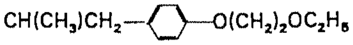
In all experiments tracheal pressure, soleus muscle contractions, arterial blood pressure and heart rate were recorded simultaneously. Completed preparations were left to stabilize for 60 min before use.

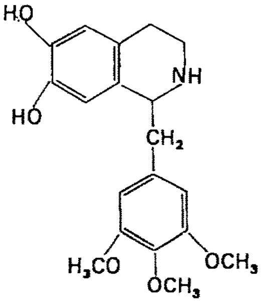
Studies with agonists

(−)-Isoprenaline was used as the reference drug in each experiment. (−)-Isoprenaline and the test β-adrenoceptor agonist were administered alternately, beginning with doses selected for each drug to produce near-threshold responses and using 3-fold increases in dose in each case. Not more than four dose-levels of each drug were administered; the interval between doses was at least 30 min and 6–8 h were required to complete the experiments. This experimental protocol was adopted so as to avoid the desensitization which has been observed with β-adrenoceptor agonists, particularly on blood pressure responses (Daly, Farmer & Levy, 1971). The β-adrenoceptor agonists were injected 1 min before 5-HT challenge and then further 5-HT doses given until constant responses were again obtained. The largest inhibitory effect was always observed at the 1 min interval. Log dose-effect curves, using the maximum observed responses, were constructed for percentage inhibition of 5-HT-induced bronchoconstriction, for decrease in tension developed during a submaximal tetanus (g), for decrease in diastolic blood pressure (mmHg) and for increase in heart rate (beats/minute). Equipotent doses for (–)-isoprenaline and test β-adrenoceptor agonist were calculated in each experiment from about the mid-point of the straight-line portion of dose-effect curves. In practice, this corresponded to 50% inhibition of the 5-HT response, 200–400 g decrease in soleus muscle tension, 20–40 mmHg decrease in diastolic blood pressure and 20–40 beats/min increase in heart rate.

Table 1 Chemical structures of the β-adrenoceptor agonists studied



	*R*1	*R*2	*R*3	*R*4
Catecholamines				
Isoprenaline	OH	OH	H	$CH(CH_3)_2$
Resorcinol analogues				
Orciprenaline	OH	H	OH	$CH(CH_3)_2$
Fenoterol	OH	H	OH	
Terbutaline	OH	H	OH	$C(CH_3)_3$
Saligenin analogues				
AH 3021	CH_2OH	OH	H	$CH(CH_3)_2$
Salbutamol	CH_2OH	OH	H	$C(CH_3)_3$
AH 4553	CH_2OH	OH	H	
Salmefamol	CH_2OH	OH	H	
AH 10013	CH_2OH	OH	H	
AH 7616	CH_2OH	OH	H	
AH 12561	CH_2OH	OH	H	
Other compounds				
AH 4941	$CH_2NHCONH_2$	OH	H	$C(CH_3)_3$
AH 4644	CH_2NHCOH	OH	H	$C(CH_3)_3$
AH 4325	$CH_2NHSO_2CH_3$	OH	H	$C(CH_3)_3$
Soterenol	$NHSO_2CH_3$	OH	H	$CH(CH_3)_2$
Trimetoquinol				



Studies with antagonists

Log dose-effect curves for (—)-isoprenaline were determined on each parameter before and from 15 min after administration of propranolol or practolol. The (—)-isoprenaline dose-effect curves took 1.5–2 h to complete. The antagonist was given at intervals of 2–2.5 h in a cumulative dosing schedule. Three or four dose-levels of antagonist were tested in each cat.

The results were analysed by the method of Arunlakshana & Schild (1959). In each experiment and for each parameter, log [(—)-isoprenaline dose-ratio-1] was plotted against log dose antagonist (mg/kg). The regression line was fitted by eye. The slope of the regression and the dose of antagonist to produce an isoprenaline dose-ratio of 10 (DR_{10}) were estimated.

The direct effects of propranolol and practolol on 5-HT-induced bronchoconstriction, soleus muscle contractions, blood pressure and heart rate were also assessed in these experiments.

Table 2 Potency estimates for 15 β-adrenoceptor agonists relative to (—)-isoprenaline on bronchial muscle, soleus muscle, blood pressure and heart rate in the anaesthetized cat

		Mean equipotent dose			
	No. of experiments	*Inhibition of 5-HT-induced bronchospasm*	*Decrease in soleus muscle tension*	*Decrease in diastolic blood pressure*	*Increase in heart rate*
AH 4325	4	1.9 (1.1–3.7)	4.5 (2.4–8.3)	5.5 (2.6–11.6)	12.3 (9.4–16.1)
Fenoterol	5	2.5 (1.2–5.1)	2.9 (1.5–5.6)	2.1 (1.3–3.5)	6.3 (2.9–13.8)
AH 4553		2.6 (1.3–5.3)	5.3 (3.1–9.1)	5.3 (3.3–8.7)	10.9 (4.9–23.8)
Trimetoquinol	4	2.9 (1.9–4.5)	3.4 (2.5–4.6)	4.5 (1.6–13.3)	4.1 (2.4–6.9)
Soterenol	4	3.6 (1.7–7.8)	8.1 (5.9–11.2)	5.2 (4.0–6.8)	33.9 (15.6–73.7)
AH 12561	4	4.1 (1.9–8.7)	4.2 (1.8–9.9)	5.4 (4.7–6.2)	17.7 (5.2–60.5)
Salmefamol	4	4.4 (2.1–9.2)	10.3 (4.7–22.3)	7.6 (5.2–11.0)	25.9 (11.2–60.3)
Salbutamol	9	8.9 (6.4–12.3)	9.3 (7.9–11.0)	9.4 (6.6–13.6)	27.0 (19.7–37.2)
AH 4644	4	10.5 (8.9–12.5)	11.6 (8.1–16.6)	10.8 (5.9–19.5)	43.3 (30.2–62.0)
Terbutaline	5	13.6 (7.8–23.6)	23.2 (14.2–37.8)	19.7 (9.2–42.2)	112.7 (73.4–172.8)
AH 4941	4	16.6 (8.7–31.8)	29.7 (19.9–44.2)	33.5 (16.3–69.2)	74.2 (39.2–140.3)
AH 7616	4	25.7 (10.5–62.9)	125.7 (77.2–204.5)	105.4 (43.9–253.3)	871.0 (608.7–1246.5)
AH 3021	4	26.5 (10.9–64.5)	31.2 (19.8–49.2)	24.4 (8.5–70.6)	123.4 (58.9–258.2)
Orciprenaline	6	30.3 (22.1–41.7)	35.8 (27.1–45.8)	44.8 (30.7–65.5)	73.4 (44.3–121.4)
AH 10013	4	49.4 (34.5–70.6)	61.2 (20.9–179.7)	59.6 (46.5–76.4)	220.0 (171.7–281.8)

Results expressed as mean equipotent dose [(—)-isoprenaline = 1] with 95% confidence limits in brackets.

Statistical analysis

Equipotent doses quoted in the text and tables are geometric means with 95% confidence limits in brackets unless otherwise stated. Statistical comparisons were made using Students *t*-test and differences were considered to be significant when $P < 0.05$.

Drugs

The drugs used were: acetylcholine chloride (British Drug Houses); 5-hydroxytryptamine creatinine sulphate (British Drug Houses); pentobarbitone sodium (Abbott) and α-chloralose (Hopkin & Williams). The β-adrenoceptor agonists used were: (–)-isoprenaline bitartrate dihydrate (Ward-Blenkinsop); orciprenaline sulphate (Boehringer Ingelheim); fenoterol hydrobromide (Boehringer Ingelheim); terbutaline sulphate (Draco); AH 3021 hydrochloride; salbutamol base; AH 4553 anisic acid monohydrate; salmefamol base; AH 10013 base; AH 7616 acetate; AH 12561 citrate; AH 4941 fumarate; AH 4644 formamide acetate; AH 4325 acetate (Allen & Hanburys Research); soterenol hydrochloride (British Drug Houses) and trimetoquinol base (Tanabe Seiyaku). With the exception of (–)-isoprenaline all the β-adrenoceptor agonists were racemic mixtures. The chemical structures of the β-adrenoceptor agonists are shown in Table 1. The β-adrenoceptor antagonists used were (±)-practolol and (±)-propranolol hydrochloride (Imperial Chemical Industries).

Stock solutions were freshly prepared for each experiment in saline. Dilutions were stored on ice in a light-proof box. Ascorbic acid (20 μg/ml) was added to solutions of (–)-isoprenaline to inhibit oxidation. Doses are expressed in terms of the base equivalent.

Results

Agonists

Each of the 16 agonists investigated decreased 5-HT-induced bronchoconstriction, decreased the tension developed during submaximal tetanic contractions of soleus muscle, decreased diastolic blood pressure and increased heart rate in a dose-related manner. The slopes of the dose-effect curves for the 15 test agonists were parallel to those for (–)-isoprenaline on all four parameters. The effects of each drug were antagonized by propranolol (0.5 mg/kg).

Potency estimates for the test agonists relative to (–)-isoprenaline are given in Table 2. (–)-Isoprenaline was the most potent agonist in the series and its action was essentially unselective. On all four parameters the minimal effective dose for (–)-isoprenaline was 0.003–0.01 μg/kg and maximal or near maximal responses were produced by 0.3–1 μg/kg. The other agonists can be divided into three distinct categories of selectivity profile. The first category contains only trimetoquinol which, like (–)-isoprenaline, was essentially unselective. The second category contains 13 of the remaining 14 agonists. For each agonist potency was significantly lower on heart rate than on bronchial muscle, soleus muscle and blood pressure. There were only small and less consistent differences in potency between the three latter tissues (see Table 2). Part of an experiment comparing AH 4644 (one of the agonists in this category) with (–)-isoprenaline is illustrated in Figure 1 and dose-effect curves for the complete experiment are shown in Figure 2.

AH 7616 is the only agonist in the third category. As with the category two agonists it was significantly less potent on heart rate than on the other parameters, but was also significantly less potent on soleus muscle and blood pressure than on bronchial muscle (see Table 2). Because of this unique profile of selectivity, further experiments were carried out with AH 7616 in which acetylcholine (20–50 μg/kg) was used as the spasmogen instead of 5-HT. AH 7616 was 138 (72–263, $n=4$) times less potent than (–)-isoprenaline against acetylcholine-induced brochoconstriction. This estimate is significantly different from that against 5-HT but is very similar to those on soleus muscle and blood pressure (see Table 2). AH 7616 may, therefore, possess a specific 5-HT antagonist action in addition to its β-adrenoceptor agonist action.

The data in Table 2 were analysed further by means of correlation coefficients and regression coefficients. If the *rank order* of β-adrenoceptor agonist potency for the series is the same on two parameters then the correlation coefficient will be 1; if, in addition, the *equipotent doses* on the two parameters are the same then the regression coefficient will be 1. AH 7616 was excluded from the analysis because of the possibility of an additional action on bronchial muscle distorting the estimate of β-adrenoceptor agonist potency. Correlation coefficients and regression coefficients based on the results obtained with the other agonists are summarized in Table 3. There is a significant correlation for rank order of potency for each pair of parameters compared. This is apparent also from an inspection of Table 2; for example, as potency against 5-HT-induced bronchoconstriction decreases, potency on the other three parameters also tends to decrease. In contrast, the regression coefficients fall into two distinct groups. In Group 1, regression coefficients are close to 1, whereas in Group 2 they are much larger than 1. The reason for this difference is that Group 2 consists of comparisons of heart rate with the other three parameters and, as described above, 13 of the 15 agonists used in the analysis were significantly less potent on heart rate than on the other parameters.

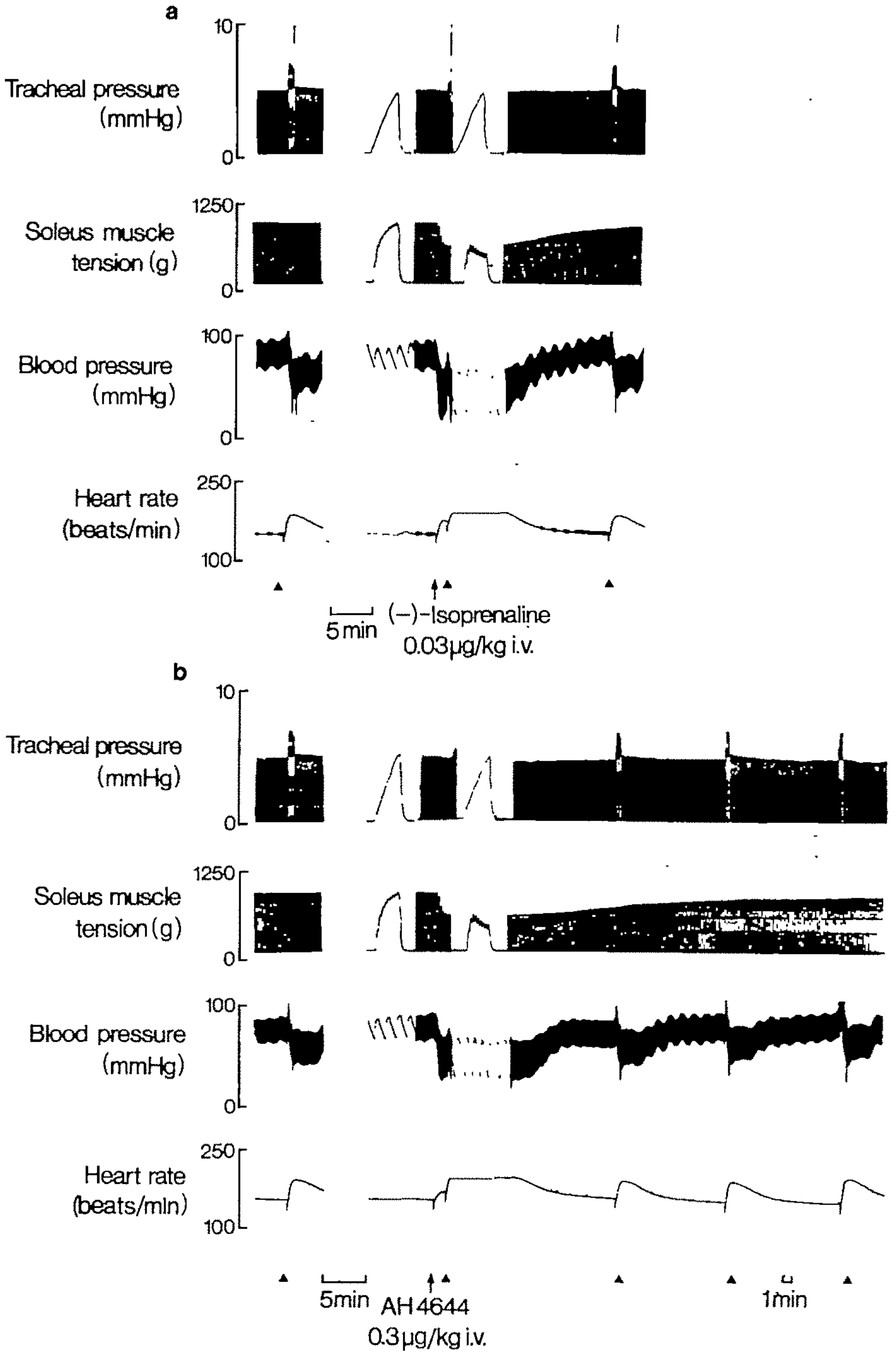


Figure 1 Anaesthetized cat, 2.2 kg ♂. Effects of (a) (—)-isoprenaline and (b) AH 4644 on 5-HT-induced bronchoconstriction, submaximal tetanic contractions of the soleus muscle, arterial blood pressure and heart rate. In each trace the paper speed was temporarily increased from 5 mm/min to 10 mm/sec before administration and at the peak effect of the β-adrenoceptor agonist to compare the degree of fusion of the soleus muscle contractions. (▲) 5-HT (5 µg/kg i.v.). The 5-HT-induced bronchoconstriction was accompanied by an increase in heart rate and a decrease in blood pressure. Note that AH 4644 had less effect on heart rate than (—)-isoprenaline at an equipotent bronchodilating dose (increases of 19 and 30 bts/min respectively), that AH 4644 was longer acting than (—)-isoprenaline and that for both AH 4644 and (—)-isoprenaline the duration of action on lung was shorter than on soleus muscle.

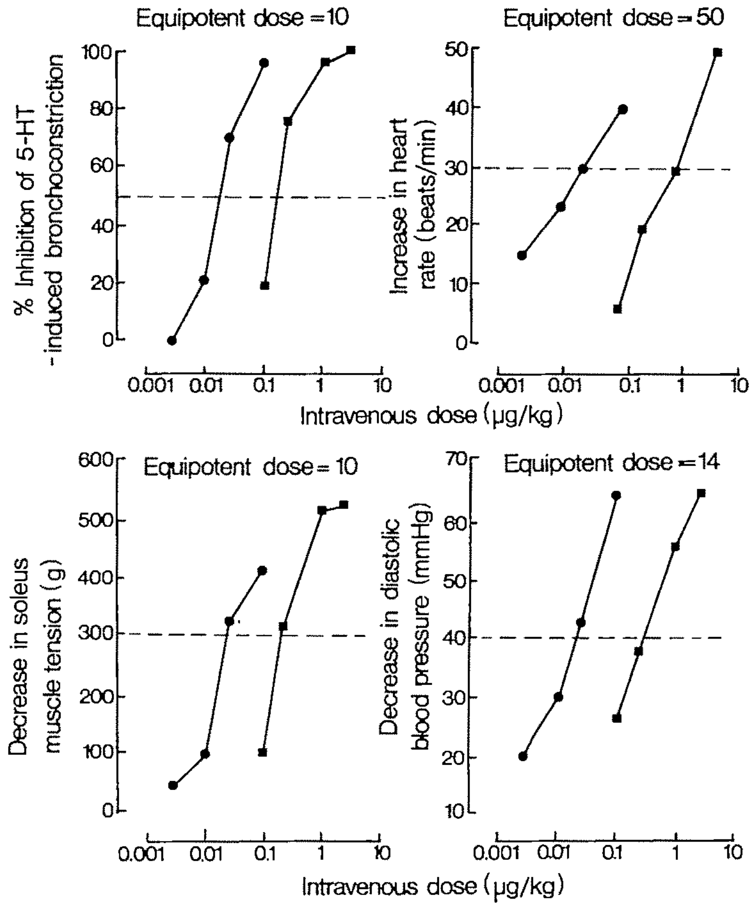


Figure 2 Anaesthetized cat, 2.2 kg ♂. Comparison of the potencies of (—)-isoprenaline (●) and AH 4644 (■). Equipotent doses ((—)-isoprenaline = 1) were calculated at ————. Part of this experiment is illustrated in Figure 1.

As well as differences in selectivity profile there were differences in time to peak effect and in the duration of action of the agonists investigated. Peak effect for 14 of the agonists was attained at 60–100 s on soleus muscle and 20–40 s on blood pressure and heart rate. Trimetoquinol and soterenol differed slightly in that peak effect was attained at 80–150 s on soleus muscle and 30–60 s on blood pressure and heart rate. The experimental design precluded precise assessment of time to peak effect on bronchial muscle. All of the test agonists were longer acting than (—)-isoprenaline and this was particularly true of trimetoquinol and soterenol. In addition, for any given agonist there were differences in duration of action between tissues. For example, the duration of the response on bronchial muscle was shorter than that on soleus muscle. This is shown for (—)-isoprenaline and AH 4644 in Figure 1.

Antagonists

In preliminary experiments, dose-effect curves for (—)-isoprenaline were obtained on bronchial muscle, soleus muscle, blood pressure and heart rate at 1.5 h intervals over a period of 6 hours. The sensitivity of individual cats ($n=4$) to (—)-isoprenaline varied by less than 2-fold over this period.

Effects of propranolol and practolol on sensitivity to (—)-isoprenaline

The results are summarized in Table 4. Propranolol (0.03, 0.1, 0.3 and 1 mg/kg), caused parallel displacements to the right of the (—)-isoprenaline dose-effect curves on all four parameters. The antagonist potency of propranolol was almost identical on bronchial muscle, soleus muscle and blood pressure but very slightly lower on the heart. Practolol (3, 10 and 30 mg/kg), also caused parallel displacements to the right of the (–)-isoprenaline dose-effect curves on all four parameters but, in contrast to propranolol, was 10–12 times more potent on the heart than on bronchial muscle, soleus muscle and blood pressure. There was no significant difference in the antagonist potency of practolol on the latter three parameters.

Partial agonist activity of practolol

Practolol (3 mg/kg), increased heart rate by 20 beats/min (range 10–29 beats/min, $n=6$), the response returning to control levels in 60–80 minutes. Administration of propranolol (5 mg/kg), during the practolol tachycardia resulted in a prompt return to control levels or below. A typical experiment is illustrated in Figure 3a. Higher doses of practolol (10–100 mg/kg) had little or no effect on heart rate, but the 30 mg/kg dose produced a slight reduction and the 100 mg/kg dose a marked reduction in soleus muscle contractions lasting for 3–8 minutes. Propranolol (5 mg/kg), had no effect on, or potentiated the soleus muscle response to practolol (Figure 3b). Thus, it would appear that the effect of practolol on heart rate arises from its partial agonist action, whereas its effect on soleus muscle does not.

Propranolol had no detectable partial agonist activity in doses up to 1 mg/kg.

Discussion

In this study the actions of a wide variety of β-adrenoceptor agonists and of two β-adrenoceptor antagonists have been determined in a single species simultaneously on bronchial muscle, soleus muscle, blood pressure and heart rate. The cardiovascular and soleus muscle actions of three of the agonists – salbutamol, orciprenaline and trimetoquinol – have been described previously (Bowman & Nott, 1970; Apperley & Daly, 1972; Houston & Rodger, 1974). Our results agree closely with those of the previous reports.

Our study was designed to provide information on two inter-related questions. First, is it possible to separate the bronchodilating and tremor-enhancing actions of β-adrenoceptor agonists? Our results show that sensitivity on bronchial and soleus muscle is similar for each of the 16 agonists investigated. Corresponding studies in the anaesthetized cat with the β-adrenoceptor agonists rimiterol (Bowman & Rodger, 1972), MJ 9184-1 (Gwee, Nott, Raper & Rodger, 1972) and isoetharine (Rodger, 1973)

Table 3 Correlation coefficients and regression coefficients for β-adrenoceptor agonist activity in the anaesthetized cat

Comparison	*Correlation coefficient*	*Regression coefficient*
Group 1		
Bronchial muscle *v* soleus muscle	0.98*	1.20 (1.06–1.35)
Bronchial muscle *v* blood pressure	0.96*	1.21 (0.99–1.43)
Soleus muscle *v* blood pressure	0.98*	1.00 (0.87–1.14)
Group 2		
Heart *v* bronchial muscle	0.97*	4.05 (3.02–5.08)
Heart *v* soleus muscle	0.99*	3.38 (2.67–4.09)
Heart *v* blood pressure	0.91*	3.01 (1.94–4.09)

Values estimated from data in Table 2. 95% confidence limits in brackets. AH 7616 omitted.

Bronchial muscle – inhibition of 5-HT-induced bronchoconstriction. Soleus muscle – decrease in tension developed during sub-maximal tetanic contraction. Blood pressure – decrease in diastolic blood pressure. Heart – increase in heart rate.

* Significant correlation ($P<0.001$).

Group 1 regression coefficients are not significantly different from one another.

Group 2 regression coefficients are not significantly different from one another.

Group 1 regression coefficients are significantly different from Group 2 regression coefficients.

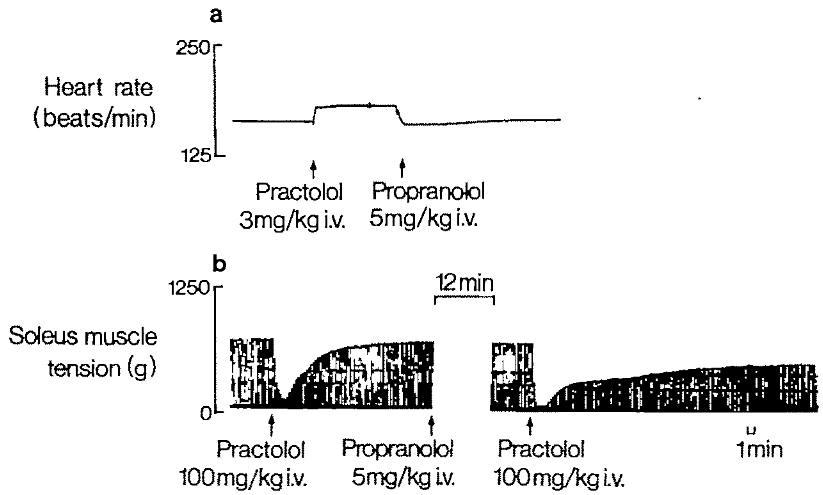


Figure 3 Anaesthetized cat, 3 kg ♂. The effect of practolol on (a) heart rate and (b) soleus muscle contractions. Note that propranolol (5 mg/kg i.v.) blocked the increase in heart rate to practolol but potentiated the soleus muscle response.

produced the same finding. If the cat soleus muscle preparation is a predictive model for enhancement of physiological tremor in man, as has been suggested (Bowman & Nott, 1970) then all of the β-adrenoceptor agonists examined to date would be expected to enhance physiological tremor and cause bronchodilatation over the same dose-range when administered systematically. It is noteworthy that in the two studies reported in man in which this question has been examined in detail, the bronchodilator actions of salbutamol, terbutaline and rimiterol were accompanied by enhanced physiological tremor (Watson & Richens, 1974; Marlin & Turner, 1975). We therefore agree with the previous conclusions (Bowman & Nott, 1970; Bowman & Rodger, 1972) that it may prove impossible to separate the bronchodilating and tremor-enhancing properties of β-adrenoceptor agonists except by giving the drugs by inhalation.

One agonist in our series, AH 7616, did appear at first sight to differentiate bronchial and soleus muscle β-adrenoceptors but it was shown subsequently that a more likely explanation of the observed selectivity is the presence of an additional 5-HT-antagonist action. The combination of potent β-agonist and 5-HT antagonist actions in one drug appears to be novel. It is, however, difficult to envisage any advantage for AH 7616 over conventional β-adrenoceptor agonists in the treatment of asthma since it is unlikely that 5-HT contributes to the underlying bronchospasm.

The second question is: how do our results relate to the problem of the sub-classification of β-

Table 4 Antagonist activity of propranolol and practolol against bronchial muscle, soleus muscle, blood pressure and heart rate responses to (−)-isoprenaline in the anaesthetized cat

Antagonist	*Inhibition of 5-HT-induced broncho-contriction*		*Decrease in tension of submaximal tetanus of soleus muscle*		*Decrease in diastolic blood pressure*		*Increase in heart rate*	
	DR_{10}	*Slope*	*DR_{10}*	*Slope*	*DR_{10}*	Slope	*DR_{10}*	*Slope*
Propranolol (*n*=6)	0.13 (0.08–0.19)	1.07 (0.9–1.3)	0.13 (0.07–0.3)	1.04 (0.9–1.1)	0.11 (0.07–0.2)	1.04 (0.9–1.1)	0.23 (0.18–0.3)	1.06 (0.8–1.3)
Practolol (*n*=6)	25.2 (10.8–58.8)	0.96 (0.7–1.2)	22.4 (14.2–35.4)	1.08 (0.9–1.4)	27.8 (23.7–32.5)	0.96 (0.9–1.1)	2.3 (1.6–3.2)	0.9 (0.8–1.0)

DR_{10} – dose of antagonist (mg/kg i.v.) to produce an isoprenaline dose-ratio of 10.
Slope – of regression obtained from plot of log (isoprenaline dose-ratio – 1) on log antagonist dose (mg/kg i.v.).
95% confidence limits are in brackets. *n*=no. of cats.

adrenoceptors? Lands' classification is widely used, but several workers have found difficulty in accommodating their results within a simple β_1/β_2 adrenoceptor framework (see Furchgott, 1972 and Daly, Flook & Levy, 1975 for references), and Barrett (1973) described Lands' classification as 'an over simplification that is neither valid nor useful'. There are two standard procedures for receptor classification, namely the determination of relative potencies in a series of agonists and determination of the potencies of competitive antagonists (Furchgott, 1972); both have been used in the present study. When compared with (−)-isoprenaline, 13 of the 15 agonists examined were clearly less potent on cardiac muscle than on bronchial, soleus or vascular muscle. Practolol showed the opposite selectivity, being some 10–12 times more potent as an antagonist on cardiac muscle than on bronchial, soleus or vascular muscle. These complementary findings provide strong support for the dual β-adrenoceptor concept of β_1-adrenoceptors in cardiac muscle and β_2-adrenoceptors in bronchial, soleus and vascular muscle. Furthermore, the similar potency of each drug, whether agonist or antagonist, in the latter three tissues points to the homogeneity of the β_2-adrenoceptor sub-type. This evidence is particularly striking when it is recalled that the experiments were carried out in intact animals and therefore under conditions which could give rise to differences in potency even though the receptors involved were identical (Furchgott, 1972; Daly *et al.*, 1975). We conclude, therefore, that Lands' classification is both valid and useful, at least in the four tissues examined. This conclusion is in line with those of previous investigations carried out in this laboratory with β-adrenoceptor agonists and antagonists in anaesthetized dogs (Daly *et al.*, 1971, 1975) and guinea-pigs (Apperley & Levy, 1975). We cannot, of course, discount the possibility that further differentiation of β-adrenoceptors might be achieved with novel β-adrenoceptor agonists or antagonists, perhaps through combination with as yet unidentified different exosites in the different tissues (Brittain, Jack & Ritchie, 1970).

Several other points worthy of comment emerge from our study. Lands' classification was derived from data obtained with a series of catecholamines (Lands *et al.*, 1967). Because criticisms of the classification arose from results obtained with non-catecholamine β-adrenoceptor agonists or with β-adrenoceptor antagonists it has been suggested that adrenoceptor classification be undertaken primarily with catecholamines and that non-catecholamine agonists or antagonists are useful only in a corroborative role (Arnold & McAuliff, 1971; Arnold, 1972; Grana, Lucchelli & Zonta, 1974). However, no such restriction need be applied to our results; Lands' classification is supported by data obtained with non-catecholamine β-adrenoceptor agonists and with β-adrenoceptor antagonists.

The existence of sub-types of β-adrenoceptor was indicated in the present study by comparisons of *relative* potency in the agonist series (regression coefficient analysis, Table 3), but not by comparisons of *rank order* of potency (correlation coefficient analysis, Table 3). O'Donnell & Wanstall (1974) also found this to be so for analogues of isoprenaline and orciprenaline on guinea-pig atria, trachea and hind limb vasculature. Thus, comparison of rank order of potency, first used to differentiate α- and β-adrenoceptors (Ahlquist, 1948) and then β_1- and β_2-adrenoceptors (Lands *et al.*, 1967) is not a completely reliable method for differentiating receptors; its success appears to depend on the particular agonists used.

Other workers have found that the selectivity of β_2-adrenoceptor agonists is lower in the cat than in other laboratory species, for example the guinea-pig (Bowman & Rodger, 1972; Rodger, 1973; Houston & Rodger, 1974; Davey, Malta & Raper, 1974). Our results are consistent with this conclusion. Furthermore, the β_2-adrenoceptor agonists salbutamol and MJ 9184-1 are weak partial agonists compared to isoprenaline in guinea-pig isolated cardiac muscle (Farmer, Kennedy, Levy & Marshall, 1970; Davey *et al.*, 1974) but are potent full agonists in cat isolated cardiac muscle (Cornish & Miller, 1975; Davey *et al.*, 1974). These findings led to the suggestion that β-adrenoceptors in cat and guinea-pig may be different (Davey *et al.*, 1974) but an alternative explanation, consistent with the available evidence, can be envisaged. There is no reason to suppose that the relationship between stimulus, as defined by Stephenson (1956), and final response is the same for all organs, or for the same organ in different species. If there are significant differences in this regard then comparison of the relative potency of agonists with unequal efficacies (Stephenson, 1956) would reveal differences even though the receptors concerned were identical (Furchgott, 1972; Jenkinson, 1973). In particular, an agonist with a low efficacy would be relatively less potent in a tissue in which a high stimulus was necessary for threshold response than one in which a low stimulus was necessary. Salbutamol and MJ 9184-1 must have lower efficacies than isoprenaline in cardiac muscle, since they are partial agonists in guinea-pig atria. This is also true for several of the other β_2-adrenoceptor agonists examined in our study (unpublished observations). The species differences could therefore be explained by postulating that the stimulus necessary for threshold response is higher in guinea-pig than in cat cardiac muscle. Two further observations, both involving practolol, are in accord with this idea. First, practolol possesses weak partial agonist activity. This activity, however, is much more obvious in cat than in guinea-pig cardiac muscle (Kaumann, A.J. & Blinks, J.R., personal communication). Second, the antagonist potency of a drug is determined only by its affinity for the receptor and should therefore be independent of differences in the stimulus-response relationship. Hence, it is instructive to compare β-adrenoceptor antagonist potency in guinea-pig and cat cardiac muscle. These data are available for practolol and show that the pA_2 against isoprenaline in guinea-pig cardiac muscle (6.76, Drew & Levy, 1972) is virtually identical with that in cat cardiac muscle (6.55, Cornish & Miller, 1975), indicating that the β-adrenoceptors involved are the same. Thus, our hypothesis has the advantage of reconciling apparently conflicting results with β-adrenoceptor agonists and antagonists.

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BLOCKADE BY BURIMAMIDE OF THE RESTORATIVE EFFECT OF HISTAMINE IN TETRODOTOXIN-TREATED HEART PREPARATIONS

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1 In isolated heart preparations in which fast sodium channels were blocked by tetrodotoxin (2–4×10^{-5} M), excitability was restored by histamine (6×10^{-6} M to 10^{-5} M).

2 This effect was antagonized by EDTA (2×10^{-6} M), D_{600} compound (0.5 μg/ml) and the H_2-receptor antagonist, burimamide (2×10^{-4} M).

Introduction

The positive inotropic effect of histamine has been largely investigated from the point of view either of the biochemical changes, or of the type of receptor involved in the mechanical response (McNeill & Muschek, 1972; Poch, Kukovetz & Scholz, 1973; McNeill & Verma, 1974; Moroni, Ledda, Fantozzi, Mugelli & Mannaioni, 1974).

On the other hand, few results of electrophysiological studies on the mechanism of the inotropic response of the amine are at present available (Houki, 1973; Watanabe & Besch, 1974). In the present study we attempted to demonstrate that histamine is able to activate the slow inward calcium current in heart preparations in which fast sodium channels were blocked by tetrodotoxin, and that this effect of the amine is mediated by H_2-receptors.

Methods

Ventricular strips of guinea-pig heart were placed in a 15 ml chamber containing Tyrode solution of the following composition (mM): Na^+ 149.3, K^+ 2.7, Ca^{++} 1.8, Mg^{++} 1.05, Cl^- 145.4, HCO_3^- 11.9, $H_2PO_4^-$ 0.4, D glucose 5.6. The solution was aerated with a gas mixture of 97% O_2 and 3% CO_2. The pH of the solution was 7.5 and the temperature kept constant at 32°C. The preparations were paced at 150 stimuli/min, and transmembrane action potentials were recorded by microelectrodes filled with 3 M KCl (resistance about 10 megohms).

The first derivative of the depolarization phase was obtained by a differentiator (operational amplifier) with a linear output between 100 and 1000 V/s. The preparations were first treated with tetrodotoxin (2–4×10^{-5}M) and became inexcitable in a few minutes. At this time the stimulation rate was reduced to 30/min and the stimulus strength and duration were doubled. Increasing concentrations of histamine (histamine dihydrochloride, Calbiochem) were then added to the perfusion fluid at 10 min intervals, and the minimal dose able to restore the excitability was checked.

The following drugs were used in order to control the specificity of the observed effect: ethylenediaminetetraacetic acid (EDTA, Merck), D_{600} hydrochloride (kindly supplied by Knoll) and burimamide (kindly supplied by Smith, Kline & French Laboratories).

Results

Histamine at the higher concentration used (10^{-5} M) was unable to restore the excitability of tetrodotoxin-treated preparations when the original stimulus parameters were used. However if the stimulus strength and duration were doubled and the stimulation rate was reduced from 150 to 30/min, electrical activity as well as contraction were restored by high concentrations of histamine (6×10^{-6} M in one experiment and 10^{-5} M in three experiments).

In two experiments it was observed that such a restoration of excitability was blocked by D_{600} (0.5 μg/ml; Figure 1) or by EDTA (2×10^{-6} M).

Moreover burimamide (2×10^{-4} M), when applied after histamine, constantly abolished the restoration of excitability induced by the amine (Figure 2).

Discussion

Demonstration that the restoration of action potentials by histamine in tetrodotoxin-treated preparations is dependent on activation of the slow calcium inward current was obtained in the present study by the

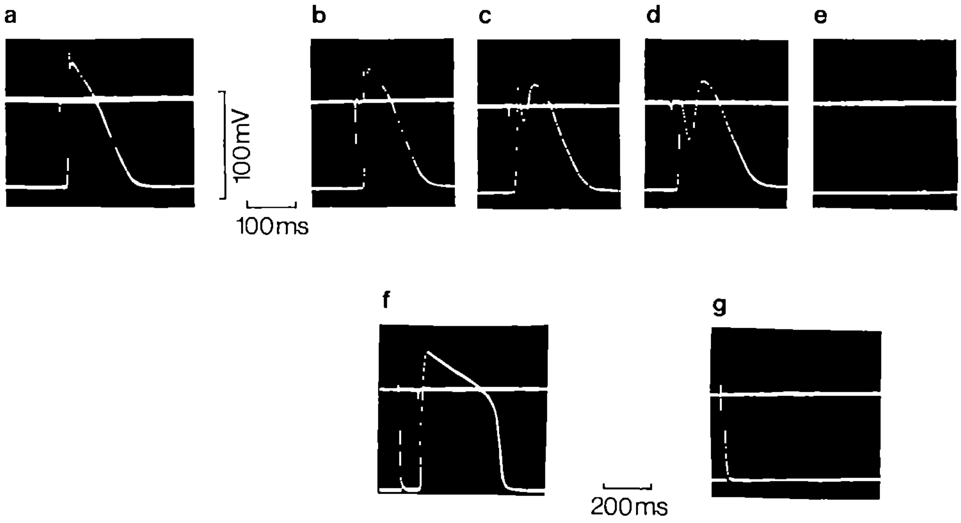

Figure 1 Transmembrane action potentials of strips of guinea-pig ventricular heart muscle. *Upper row*: Stimulation rate 150/min; (a) control; (b), (c), (d) and (e) at 60, 90, 120 and 180 s respectively after treatment with tetrodotoxin (2×10^{-5} M). *Lower row*: Stimulation rate 30/min; (f) 10 min after histamine (10^{-5} M) in the presence of tetrodotoxin; (g) 10 min after D_{600} (0.5 µg/ml) in the presence of histamine and tetrodotoxin as in (f). Incubation temperature, 32°C.

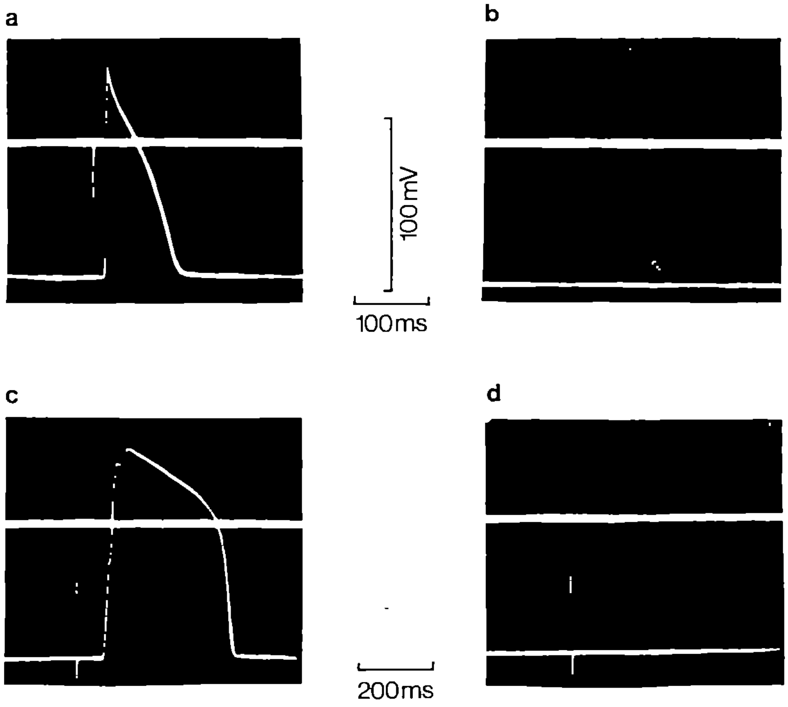

Figure 2 Transmembrane action potentials of strips of guinea-pig ventricular heart muscle. *Upper row*: Stimulation rate 150/min; (a) control; (b) 3 min after treatment with tetrodotoxin 2×10^{-5} M. *Lower row*: Stimulation rate 30/min; (c) 10 min after histamine (10^{-5} M) in the presence of tetrodotoxin; (d) 10 min after burimamide (2×10^{-4} M) in the presence of histamine and tetrodotoxin as in (c). Incubation temperature, 32°C.

chelation of extracellular calcium with EDTA and by the use of the specific inhibitor of calcium transmembrane influx D_{600} (Kolhardt, Bauer, Krause & Fleckenstein, 1972).

Therefore it is possible to conclude that histamine was able to increase calcium inward current in myocardial fibres, as previously shown by Houki (1973) in potassium-depolarized heart preparations.

Moreover the H_2-receptor blocking drug burimamide (Black, Duncan, Durant, Ganellin & Parsons, 1972) effectively antagonized the increase in calcium influx induced by activation of histamine receptors.

This observation is in good agreement with the well-known antagonism of the effects of histamine on cardiac muscle and cyclic AMP levels by H_2-receptor blocking drugs (Poch *et al.*, 1973; McNeill & Verma, 1974; Moroni *et al.*, 1974).

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INVESTIGATIONS TO CHARACTERIZE A NEW ANTI-ARRHYTHMIC DRUG, ORG 6001, INCLUDING A SIMPLE TEST FOR CALCIUM ANTAGONISM

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1 The compound Org 6001 (3α-amino-2β-hydroxy-5α-androstan-17-one hydrochloride) was found in recent experiments to exhibit anti-arrhythmic activity. Evidence is presented in this paper concerning its mode of action.

2 Org 6001 was 1.8 times more potent than procaine as a local anaesthetic on desheathed frog nerve.

3 Org 6001 had no effect on the resting potential of isolated cardiac muscle of rabbit, but greatly reduced the maximum rate of depolarization (MRD). The action potential duration (APD) was marginally prolonged in atrial and ventricular muscle.

4 Org 6001 preferentially shortened APD in that part of the Purkinje system in which APD is normally longer than elsewhere, so that APD became more uniform throughout the ventricular conducting system.

5 Org 6001 did not block chronotropic responses to isoprenaline in atrial muscle.

6 Org 6001 had only a small negative inotropic effect in atrial muscle, and did not reduce the positive inotropic effect of raised calcium concentrations.

7 The effect of Org 6001 on MRD was reduced by lowering the external K^+ concentration.

8 It is concluded that Org 6001 is an anti-arrhythmic drug of the first class (local anaesthetic type), and within this group is of a sub-class more closely related to lignocaine than to quinidine.

Introduction

There is still a need in the cardiologist's armamentarium for an anti-arrhythmic drug, rapidly and reliably absorbed when given orally, and with low toxicity and long half-life, for maintenance or prophylactic therapy. Several new compounds have been introduced recently which might fill this role (e.g. mexillitine, Allen, Kofi Ekue, Shanks & Zaidi, 1970; Singh & Vaughan Williams, 1972a) and some established drugs are being re-assessed. The structure of Org 6001 (Figure 1) is quite unlike that of any other anti-arrhythmic drug, and it was naturally of interest to discover whether it might have a novel mode of action also.

There are four main ways in which currently known anti-arrhythmic drugs may act, several compounds possessing more than one of these classes of action (Vaughan Williams, 1970; 1974; 1975). The first consists of a direct membrane 'stabilization', which may be measured in a number of ways (raised electrical threshold, slowed conduction velocity, reduced frequency at which stimuli can be followed), but high concentrations are required to demonstrate these effects, and the most sensitive test is measurement of the maximum rate of depolarization (MRD) with intracellular electrodes. Compounds with this class 1 action on cardiac muscle also have a variable local anaesthetic activity on nerve. The second class of action is anti-sympathetic, by competitive receptor blockade, reduction of transmitter release, or post-synaptic interference with responses to adrenergic stimulation. The third class of action consists of a prolongation of the cardiac action potential, again most reliably recorded with intracellular electrodes, although a large prolongation of ventricular action potentials can usually be detected as a lengthened Q-T interval on the electrocardiogram. Finally, the fourth class of action is an interference with inward calcium currents, but there is, as yet, no simple method for measuring this effect, which is still not as fully authenticated as the other three (Singh & Vaughan Williams, 1972b).

The present investigation has been devoted to determining which, if any, of these actions is exhibited by Org 6001, and a simple test, which gives a positive result with verapamil, has been devised to measure calcium antagonism.

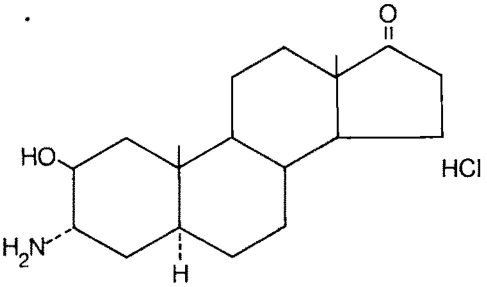

Figure 1 Structure of 3α-amino-2β-hydroxy-5α-androstan-17-one hydrochloride (Org 6001) mol. wt. 341.9, pKA 8.0.

Methods

Atrial recording

Atria removed from rabbits weighing approximately 1 kg of either sex were set up for the recording of mechanical and electrical activity as previously described (Vaughan Williams, 1958; Szekeres & Vaughan Williams, 1962). The atria were suspended in modified Locke solution gassed with 95% O_2 and 5% CO_2 at 32°C. The solution had a composition of (mM) NaCl 125, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 25, glucose 11, and had a pH of 7.4. During impalements with micro-electrodes the atria were paced at a frequency 10% above the spontaneous frequency by square stimuli, 1 ms duration, strength × 2 threshold. Records were made after 30–60 min exposure to each drug concentration. Contractions were recorded with an RCA 5734 transducer and intracellular potentials with 3M-KCl-filled glass micropipettes.

Ventricular recording

Hearts from stunned 2 kg rabbits of either sex were removed and placed in oxygenated Locke solution at 35°C. Longitudinal cuts, parallel to the septum, were made along the anterior and posterior interventricular grooves. The tricuspid chordae tendiniae were severed at their insertions and the right ventricular free wall was reflected from the septum. The right bundle branch was cut at the level of the membranous septum, and a block of septal muscle (3–5 mm) containing the right bundle was cut free. The bundle becomes a clear strand at the base of the anterior papillary muscle, with two or more branches being inserted into the right ventricular free wall. This portion of the wall was also cut free and the completed dissection was transferred to the recording bath, and comprised: (1) proximal His bundle; (2) right bundle; (3) anterior papillary muscle; (4) false tendons, being the sole connection to (5) the endocardial portion of the free right ventricular wall into which (4) were inserted. The preparation was pinned to a silastic base and superfused with 10 ml/min oxygenated Locke solution at 35°C ± 0.2°C. A bipolar silver stimulating electrode was placed on the proximal remnant of the His bundle. Microelectrode records were taken near the stimulating electrode and at successive intervals of 1–2 mm distal to it, along the His bundle, proximal right bundle, false tendon and ventricular muscle. The region of the conducting system with the longest action potential duration was identified, and one microelectrode was positioned 1 cm proximal to it, and another 2–3 mm distal or in the ventricular muscle itself. The preparation was paced with twice threshold stimuli (2 ms, at 1–1.6 Hz) from the His bundle or proximal right bundle, and every sixth stimulus was followed by an additional ('premature') stimulus after a variable interval shorter than the basal cycle length.

Local anaesthesia

Frog sciatic nerves were stripped of their sheaths and mounted in a three-compartment perspex bath. Cotton wool soaked in frog Ringer solution was placed in the two outer chambers so that the segments of sciatic nerve in these chambers were in an atmosphere of moist air. The central chamber was filled with frog Ringer solution. In one of the outer chambers the nerve was in contact with a pair of platinum stimulating electrodes, in the other, it was in contact with recording electrodes. An indifferent earthing electrode was inserted into the central chamber. Nerves were stimulated with supramaximal pulses (5–10 V) of 1 ms duration and action potentials were displayed on an oscilloscope.

Drugs were added to the central chamber and left in contact with the nerve until a maximal effect on the fastest wave of the action potential was observed. The frog Ringer solution had the following composition: (mM) NaCl 120, KCl 1.88, $CaCl_2$ 1.08, $NaHCO_3$ 2.38 and was kept at a pH of 7.5 by the addition of Tris buffer (0.104 M) 10 ml per litre.

Drugs used were Org 6001 (Organon); isoprenaline sulphate (Burroughs Wellcome); procaine hydrochloride (BDH). Measurements have been presented as means ± s.e., and the significance of differences has been estimated by Student's *t* test, or Student's paired *t* test.

Results

1. Measurement of direct membrane effects (Class 1 action)

Local anaesthesia of frog nerve. Org 6001 at pH 7 had a local anaesthetic potency on frog sciatic nerve

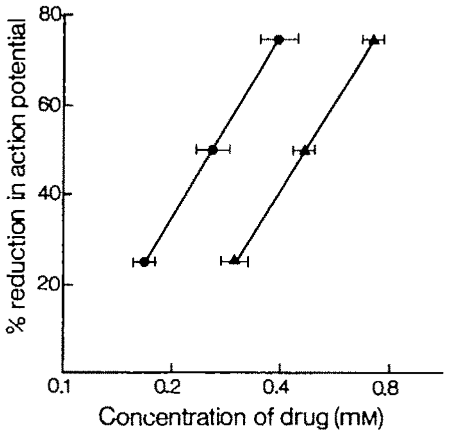

Figure 2 Local anaesthetic activity of Org 6001 (●) on frog sciatic nerve compared with that of procaine (▲). Ordinate scale; % reduction in action potential height. Abscissa scale; concentration of drug (in mM) on logarithmic scale.

1.8 times greater than that of procaine. The mean results of 8 experiments are shown in Figure 2, the ED_{25}, ED_{50} and ED_{75} having been interpolated from each individual dose-response curve to obtain the means. Not only was Org 6001 more potent than procaine, it also had a much slower onset and longer duration of action. Recovery from concentrations of Org 6001 producing greater than a 50% reduction of action potential height was never complete on washout, implying some neurotoxicity (i.e. irreversible inactivation of channels for inward Na-current).

Electrical threshold, conduction velocity and maximum driven frequency. The lowest concentration of Org 6001 studied (2.9 μM), had no effect on the electrical threshold of rabbit isolated atria, but higher concentrations (11.7 and 46.8 μM) caused significant increases. Similarly, conduction velocity was unaffected by the lowest concentration, but was significantly slowed by the higher concentrations. The maximum frequency at which the atria would follow a stimulus was, however, reduced even at the lowest concentration of Org 6001. These results are summarized in Table 1.

Atrial intracellular potentials. Measurements of various parameters of atrial intracellularly recorded potentials are summarized in Table 2, the results having been pooled from seven experiments. Concentrations of Org 6001 up to 46.8 μM had no effect on resting potential, implying a complete absence of any inhibition of ion-pumping by the membrane. A significant fall in the maximum rate of depolarization (MRD) was apparent, however, even at the lowest concentration. The overshoot was significantly reduced by the two higher concentrations, implying direct interference with Na-inward

Table 1 Effects of Org 6001 on rate and force of spontaneous contraction, electrical threshold, conduction velocity and maximum driven frequency in rabbit isolated atria

Concentration of Org 6001 (μM)	*Spontaneous frequency*	*Contractile tension*	*Electrical threshold*	*Conduction velocity*	*Maximum driven frequency*
2.9	−6.3 ± 1.6**	+10.4 ± 4.9	+8.3 ± 4.6	−4.0 ± 2.3	−6.2 ± 2.4*
11.7	−9.9 ± 2.9**	−3.0 ± 4.3	+23.5 ± 7.4	−8.8 ± 2.6**	−10.7 ± 2.2**
46.8	−16.3 ± 2.7**	−9.9 ± 5.9	+30.9 ± 8.8**	−24.3 ± 2.7***	−24.1 ± 3.1***

Values given as % change from control. Mean ± s.e. of 7 experiments.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 2 Effect of Org 6001 on rabbit atrial intracellular potentials

Dose of Org 6001 (μM)	*No. of fibres*	*Resting potential (mV)*	*Action potential (mV)*	*MRD (V/s)*	*50% repolarization time (ms)*	*90% repolarization time (ms)*
0	42	73.5 ± 0.54	98.9 ± 1.08	94.7 ± 5.10	51.0 ± 1.0	98.5 ± 1.0
2.9	36	73.0 ± 0.81	97.8 ± 1.08	80.0 ± 4.31*	52.0 ± 1.0	99.0 ± 1.5
11.7	30	74.1 ± 1.08	92.4 ± 1.62**	61.5 ± 5.78***	50.0 ± 1.0	102.0 ± 2.0
46.8	6	74.6 ± 1.08	87.0 ± 2.7**	36.8 ± 5.30***	52.0 ± 1.0	112.5 ± 1.5***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

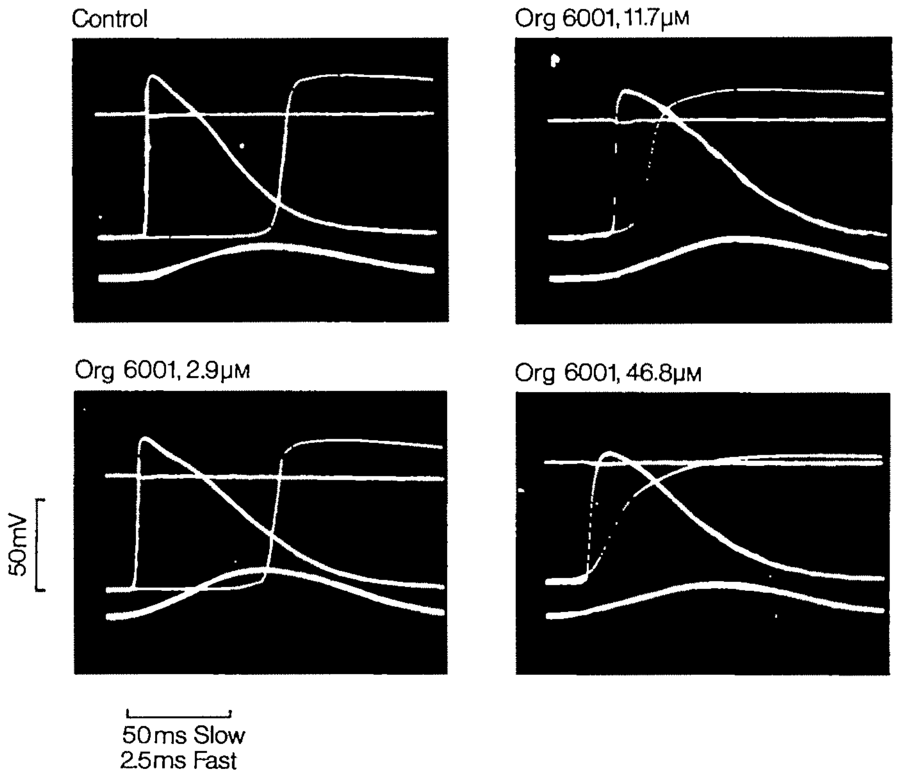

Figure 3 Effect of Org 6001 on rabbit atrial intracellular potentials. In each panel; horizontal trace, zero potential; middle traces, intracellular potentials at slow and fast sweep speeds, superimposed; lowest trace, contraction.

Table 3 Effect of Org 6001 on rabbit ventricular and Purkinje fibre intracellular potentials

	Ventricular muscle			*Purkinje fibre*		
Concentration Organon 6001 µg/ml(µM)	*Action potential height (mV)*	*MRD (V/s)*	*Action potential to 90% re-polarization (ms)*	*Number of fibres*	*Action potential height (mV)*	*MRD (V/s)*
0 (Control) No. of fibres *n*=108	105.3±1.3	308±4.1	178±2.3	*n*=130	119.1±3.2	516±11
4 µg/ml (11.7) *n*=56	103.2±3.1	*232±1.2	*208.3±1.7	*n*= 78	118.6±3.6	*349± 5.2
10 µg/ml (29.3) *n*=42	102.6±2.6	*194±1.1	*210.3±1.3	*n*= 83	116.3±3.1	*157±12.7

* $P < 0.001$ from control values.

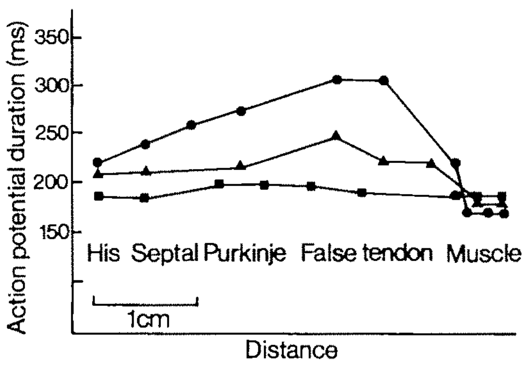

Figure 4 Effect of Org 6001 on the duration of the intracellularly recorded action potential at different parts of the conducting system, and in ventricular muscle. Ordinate scale: action potential duration (ms); abscissa scale: distance from origin of conducting system included within the preparation. (●) Controls; (▲) Org 6001 11.7 μM; (■) Org 6001 29.3 μM.

depolarizing currents, the typical class 1 effect (Figure 3). There was no prolongation of the plateau to 50% repolarization at any concentration, but there was a small but highly significant prolongation of the time to 90% repolarization at the highest concentration. Thus Org 6001 has only a weak class 3 action. Drugs exhibiting this action as a primary feature, e.g. amiodarone, (Singh & Vaughan Williams, 1970) and L 9146 (Vaughan Williams, Salako & Wittig, 1976), typically cause a greater prolongation of the plateau to 50% repolarization than to 90% repolarization.

Ventricular potentials. The His bundle was stimulated at a basal frequency of 1.0 to 1.6 Hz and intracellular recordings were made from Purkinje fibres and ventricular muscle. The resting potential was not altered by Org 6001 in any tissue. Other effects of the drug are summarized in Table 3. In contrast with atria, ventricular action potentials peak amplitudes were not affected by Org 6001 to a significant degree. The effect in ventricular muscle on MRD and action potential duration (APD) was the same as in atria, MRD being reduced and APD lengthened. The effect in Purkinje tissue was similar to that in ventricle with respect to action potential voltage and MRD, but the effect on APD depended on the region from which\the record was obtained. The normal pattern of APD in the conducting tissue is that it lengthens progressively from the bundle of His as far as the terminal chordae tendiniae, which have action potentials as much as 100 ms longer than those of the muscle into which they are inserted. Org 6001 11.7 μM shortened the duration greatly in the distal region, much less in the bundle of His. At a concentration of 29.3 μM the APD of the central and terminal sections was reduced so much that it now became as brief as that of the bundle of His itself, and since the ventricular APD was lengthened, the APD was almost uniform throughout the ventricular conduction system. The results from one experiment are depicted graphically in Figure 4 and the mean results from four preparations are presented in Table 4.

Effects on conduction. After every 6th stimulus (S_1) at the basal frequency a 7th was introduced at an interval shorter than the basal interval ('premature' stimulus, S_2). One microelectrode was positioned 1 cm proximal to the region of maximum APD, another 3–5 mm distal to it or in the ventricular muscle itself. The interval between the sixth and the premature stimulus is termed S_1–S_2;' the interval between the upstrokes of the 6th and 7th action potentials recorded by the proximal microelectrode is P_1–P_2, and the interval between the upstrokes of the records from the distal electrode is D_1–D_2. As the S_1–S_2 interval was

Table 4 Effects of Organon 6001 on action potential durations in right bundle branch

	Mean action potential duration (milliseconds)			
Drug concentration μg/ml (μM)	*His*	*Septal Purkinje*	*False tendons*	*Free wall ventricular muscle*
0 Control No. of fibres $n=108$	198.5 ± 2.3	246.8 ± 1.8	304.5 ± 4.8	178.0 ± 2.3
4 μg/ml (11.7) $n=36$	204.8 ± 1.2	*210.9 ± 1.3	*225.0 ± 3.6	*208.3 ± 1.7
10 μg/ml (29.3) $n=42$	200.3 ± 1.1	*209.8 ± 0.4	*211.8 ± 0.8	*210.3 ± 1.3

* $P<0.001$ from control values.

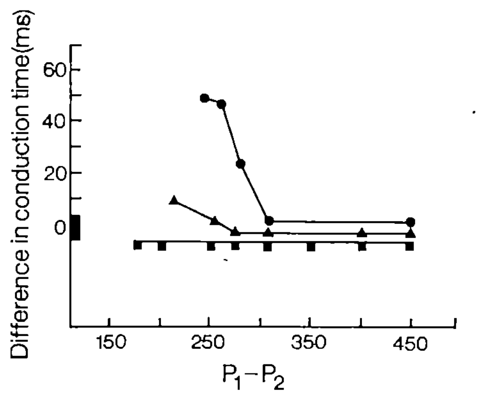

Figure 5 Effect of Org 6001 on conduction in a ventricular preparation. Ordinate scale: Difference in ms between the interval between the time of arrival of normal and premature responses recorded from the distal electrode (D_1–D_2, see text) and the interval between the arrival of normal premature action potentials at the proximal electrode (P_1–P_2). Abscissa scale: The minimal interval in ms between P_1–P_2 at which propagation still occurred of the response to the premature stimulus through to the distal electrode. In the controls D_1–D_2 was much greater than P_1–P_2, because the conduction velocity of D_2 was slowed by travelling in partially refractory tissue. Org 6001 shortened action potential duration in distal Purkinje fibres, so that propagation now occurred in less refractory tissue, and D_1–D_2 was no longer greater than P_1–P_2. (●): Controls; (▲) Org 6001 11.7 µM; (■) Org 6001 29.3 µM.

shortened, propagation of D_2 eventually failed, and the shortest interval of D_1–D_2 at which conduction was successful is the minimum propagation interval through the intervening region (which is sometimes called a 'gate' by e.g. Meyerburg, Steward & Hoffman, 1970; Wittig, Harrison & Wallace, 1973). It must be emphasized that this propagation interval is the difference between the times of arrival of action potentials in tissue distant from the stimulated region, and would include differences of conduction velocity and utilization time of the stimulus. It must, therefore, be distinguished from the 'functional refractory period', which is usually defined as the minimum interval between two stimuli, both of which are successful in eliciting action potentials propagated from the tissue stimulated.

In the control records, because APD was much longer in the conducting segment between the proximal and distal recording electrodes, when the S_1–S_2 interval was shortened P_2 impinged on refractory tissue and conduction failed, so that D_2 was absent. Before conduction failed altogether, P_2 arrived at partially refractory tissue, so that conduction velocity in the intervening segment was slowed, and D_1–D_2 was much longer than P_1–P_2 (Figure 5 (control) and Table 5).

The primary effect of Org 6001 so far demonstrated in both atrial and ventricular potentials was to reduce MRD (class 1 action) and so conduction velocity was slowed (Table 5). However, since APD in the central section was preferentially shortened by the drug, P_2 now reached tissue which had had more time to recover, so that the difference between the minimum

Table 5 Conduction in Purkinje fibres

Org 6001 concentration µg/ml (µM)	*Latency of P_1 (ms)*	*Conduction velocity Proximal segment For P_1 (m/s)*	*Conduction velocity Distal segment For D_1 (m/s)*	*Intervals (ms) S_1–S_2*	*Intervals (ms) P_1–P_2*	*Intervals (ms) D_1–D_2*
0 Controls	< 1	> 2	> 2	237	237	275
4 µg/ml (11.7)	4	0.25	0.667	198	202	215
10 µg/ml (29.3)	9	0.13	0.4	172	181	185

Latency is the interval between S_1 and P_1, and includes not only conduction time, but utilization time of the stimulus before the action potential takes off from the stimulated region. Conduction velocity for P_1 is the distance between S and P divided by the latency, and is only the 'apparent' conduction velocity because latency included utilization time. In the controls the latency was so short that it could not be measured accurately, so that conduction velocity is 'greater than' 2 m/second. The conduction velocity for D_1 is a true conduction velocity, being the distance P to D divided by the P_1–D_1 interval, and does not include utilization time. The big difference between conduction velocity for P_1 and for D_1 indicates that utilization time was greatly lengthened by the drug.

P_1–P_2 interval (at which D_2 was just conducted) and the minimum D_1–D_2 interval itself was reduced by 11.7 μM Org 6001, and abolished by 29.3 μM. These D_1–D_2 minus P_1–P_2 differences are plotted against P_1–P_2 intervals in Figure 5., but it must be emphasized that the S_1–P_1 interval itself was longer, and the conduction velocity of P_1 and D_1 was slower in the presence of the drug because of its class 1 action (Table 5). A second consequence of the class 1 action was the raising of the electrical threshold of the right bundle from 2.2 V to 3.6 V (±0.2 V) by Org 6001 11.7 μM, and to 4.5 V (±0.3 V) by 29.3 μM. Thirdly, conduction velocity of P_1 and D_1 was greatly reduced (Table 5), much more, in fact, in the Purkinje system than in atrial muscle (Table 1).

Spontaneous frequency and contractions. Drugs with direct membrane effects are often termed 'cardiodepressant', with the implication that reduction of MRD is correlated with a negative inotropic action. Though this may be true in some cases (e.g. with quinidine) it is not necessarily so, either qualitatively or quantitatively, and two drugs, papaverine (Vaughan Williams & Szekeres, 1961) and L 7810 (Bagwell, Polster & Vaughan Williams, 1973) have depressant effects on MRD in association with a positive inotropic action. A similar result was observed at the lowest concentration of Org 6001, which caused a significant reduction in MRD (Table 2) in association with an increase in contractions (Table 1). Even at the highest concentration the small reduction in contraction was not statistically significant, in contrast with the large effects on the membrane.

The spontaneous frequency of the SA node was significantly reduced by all three concentrations of Org 6001, though the effect was not as great as that on MRD.

In the ventricular preparations, spontaneous pacemaker activity was usually present (mean frequency 36 beats/min), and was always completely eliminated by the lowest concentration of Org 6001. Addition of adrenaline (50 μM) raised the spontaneous frequency of the preparations fourfold (to a mean of 136 ± 16 beats/min), but even these highly augmented rhythms were also completely abolished by the lowest concentration of Org 6001.

Effect of external K^+ concentration on MRD. The failure of nerves to recover from the effects of the higher concentrations of Org 6001, and the very large effects on MRD in rabbit atria in relation to its relative potency to procaine (× 1.8) on nerve, suggested that the activity of Org 6001 in blocking depolarizing current might be qualitatively different from the typical 'local anaesthetic' type of drug action. MRD is a function, *inter alia*, of the resting level of the potential from which the action potential 'takes off' (Weidmann, 1955). The effect of drugs with class 1 action (i.e. lignocaine, procaine, procainamide, etc.) is antagonized by low serum potassium, because the K_i/K_o ratio is thereby increased, the resting potential becomes more negative, and MRD is automatically accelerated as a result. If, however, channels for inward Na-current were irreversibly inactivated, lowering K_o, though increasing the resting potential, would no longer be able to restore MRD to normal levels.

To test this hypothesis, the effects of Org 6001 on intracellular potentials were measured in solutions containing 5.6 mM K (the normal plasma K for

Table 6 Effect of Org 6001 on rabbit atrial intracellular potentials at normal and low potassium concentrations

Dose of Org 6001 (μM)	*No. of fibres*	*K^+ (mM)*	*Resting potential (mV)*	*Action potential (mV)*	*MRD (V/s)*	*Repolarization to zero potential (ms)*	*90% Repolarization (ms)*
Control	30	5.6	78.4 ± 0.96	102.7 ± 0.90	109.7 ± 3.93	40.3 ± 1.70	102.7 ± 1.65
	12	2.8	88.7 ± 1.22	110.3 ± 1.42	122.8 ± 5.23	35.8 ± 1.91	107.5 ± 1.83
5.8	30	5.6	78.4 ± 0.89 (0)	100.0 ± 1.09 (−2.6)	83.5 ± 4.59*** (−23.9)	36.2 ± 1.38 (−10.0)	103.4 ± 1.10 (+0.75)
	30	2.8	84.9 ± 1.31 (−4.3)	108.0 ± 1.57 (−2.0)	108.2 ± 3.59* (−11.8)	35.8 ± 1.78 (+0.25)	108.8 ± 1.43 (+1.16)
23.4	30	5.6	75.1 ± 1.38 (−4.1)	93.4 ± 1.42*** (−9.0)	42.9 ± 2.58*** (−60.8)	33.1 ± 2.04* (−17.8)	99.0 ± 1.53 (−3.5)
	30	2.8	83.2 ± 1.79 (−6.1)	103.7 ± 1.02** (−6.0)	69.1 ± 2.57*** (−43.7)	34.0 ± 1.17 (−4.8)	107.7 ± 1.42 (+0.12)

Percent change from corresponding control is indicated in parentheses below the figure for each drug concentration.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

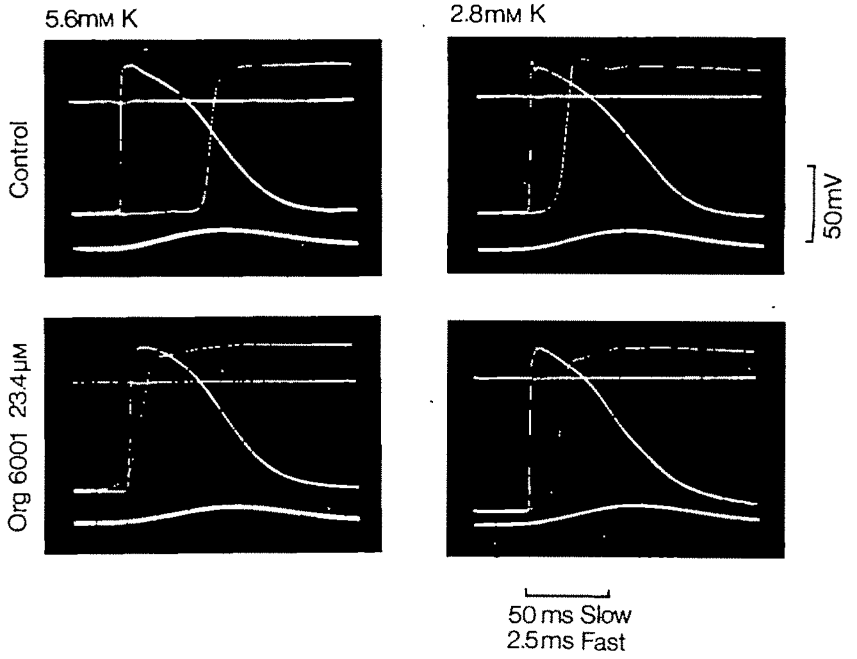

Figure 6 Influence of external potassium concentration on the effect of Org 6001 on rabbit atrial intracellular potentials. Explanation as for Figure 3. Low external potassium counteracted the effect of Org 6001, and shifted the dose-response curve, so that in low K higher concentrations of Org 6001 were required to produce the same effect as in normal K concentration.

rabbits) and 2.8 mM K. The order in which the atria were exposed to the different solutions was as follows: Controls, 5.6 mM K; controls, 2.8 mM K; Org 6001 5.8 μM at 2.8 mM K; Org 6001 5.8 μM at 5.6 mM K; Org 6001 23.4 μM at 5.6 mM K; Org 6001 23.4 μM at 2.8 mM K. Halving the external K raised the resting potential (in the absence of drug) by ten millivolts and MRD by 13 V/second. The effects of Org 6001 at the two levels of external potassium are presented in Table 6. Halving external K simply shifted the whole dose-response curve to the left. Thus at 2.8 mM K MRD in the presence of 23.4 μM Org 6001 was as fast as the control MRD at 5.6 mM K, but was nevertheless significantly slower than the control MRD at 2.8 mM K. The results do not support the view that Org 6001 irreversibly inactivated channels for inward Na current to any substantial extent. Representative examples of these results are depicted in Figure 6.

2. Adrenoceptor blocking effects (Class 2 action)

Dose-response curves for chronotropic and inotropic responses to isoprenaline by rabbit isolated atria were obtained in control solution and in the presence of 11.7 μM Org 6001. The results are plotted in Figure 7. It is apparent that Org 6001 had no blocking action on the responses to isoprenaline of the SA node. There was, however, a small but significant reduction in the inotropic responses to isoprenaline, but this would not be relevant to the arrhythmogenic action of catecholamines. It may be concluded that Org 6001 has no class 2 anti-arrhythmic action at the level of cardiac β-receptors and beyond, though the possibility of a presynaptic or central anti-sympathetic action remains to be examined.

3. Prolongation of action potential (Class 3 action)

The evidence presented in Tables 2 and 6 indicates that, so far as atrial muscle is concerned, Org 6001 had a very minor class 3 activity. In the ventricular conducting system APD is actually shortened, though some lengthening occurs in ventricular muscle, as in atrial muscle.

4. Antagonism to inward calcium currents (Class 4 action)

The suggestion that blockade of inward (depolarizing) calcium current might constitute a fourth class of anti-arrhythmic action was based upon the fact that the

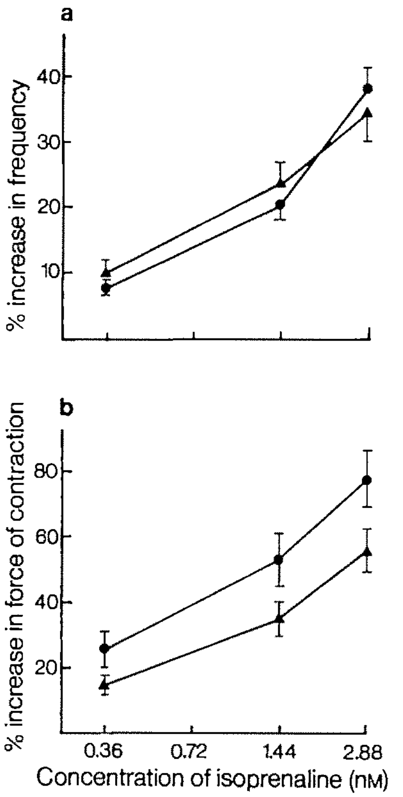

Figure 7 Effect of Org 6001 11.7 μM (4 μg/ml) on chronotropic and inotropic responses to isoprenaline. Ordinate scale; (a) % increases in heart rate and (b) in force of contraction. Abscissa scale; Concentration of isoprenaline (nM) on a logarithmic scale. The effect of Org 6001 on the inotropic responses was statistically significant by Student's paired *t* test ($P<0.05$). (●): Controls; (▲): Org 6001.

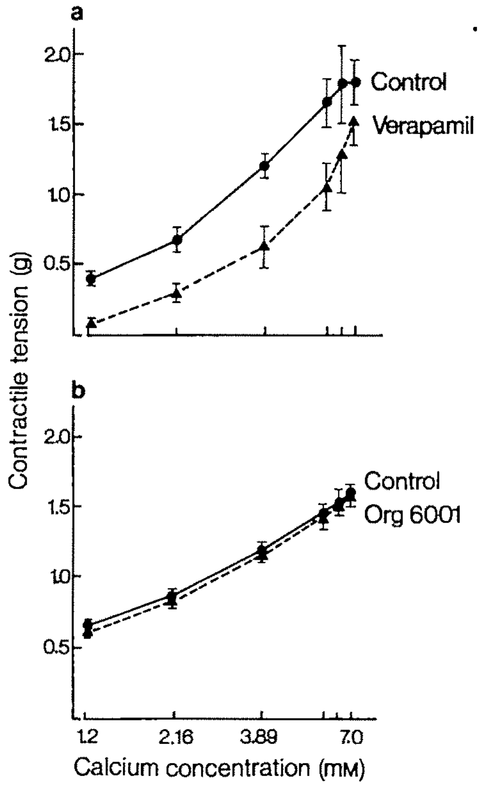

Figure 8 Effects of (a) (0.15 μg/ml) verapamil (a) and (b) Org 6001 (6 μg/ml) on the positive inotropic action of increases in Ca concentration in rabbit isolated atria. Ordinate scale: contractile force in grams. Abscissa scale: concentration (mM) of calcium in Locke solution on logarithmic scale.

drug verapamil, originally introduced as a coronary dilator, was undoubtedly anti-arrhythmic, yet had none of the three actions already described. It was strongly negatively inotropic, flattened the plateau of the action potential (Singh & Vaughan Williams, 1972b), and in high concentration reduced Ca uptake by 'sarcoplasmic reticulum' (Fleckenstein, Döring & Kammermeier, 1968), though this was disputed (Graca & van Zweiten, 1971), and its relevance to surface inward Ca-current is highly doubtful. Thus the classification of blockade of inward Ca-current as a fourth class of anti-arrhythmic action must still retain its question-mark.

The performance of voltage-clamp experiments in cardiac muscle is technically difficult, and the interpretation of the results obtained is controversial (Johnson & Lieberman, 1971). It was, therefore, decided to improvise a simple pharmacological test of the effects of external Ca concentration on contractile force in rabbit isolated atria, and to compare the effect of Org 6001 with that of verapamil. The results are presented in Figure 8. Over the range of Ca concentrations used, the relation between force and log $[Ca]_0$ is nearly linear, and over the lower part of this range verapamil caused a parallel shift to the right. The reason for which the highest Ca concentration had a tendency to 'overcome' the effect of verapamil is not known. It was quite clear, however, that Org 6001 had no effect whatever upon the relation between external Ca and contractile force, and, as thus defined, exhibited no 'calcium-antagonism' of the kind possessed by verapamil.

Discussion

A series of experiments by Marshall & Parratt (1975) indicated that Org 6001 had an anti-arrhythmic potency comparable with that of lignocaine in reducing the number of ectopic ventricular beats after ligation of a coronary artery in anaesthetized dogs. Another series of experiments (Vargäftig, Sugrue, Buckett & van Riezen, 1975) suggested that Org 6001 was one half as potent as lignocaine on desheathed frog nerve. *In vivo* tests in mice (tail-clip test) suggested that Org 6001 was 'much less effective than lignocaine as a local anaesthetic'.

It appeared at first sight, therefore, that although Org 6001 was an effective anti-arrhythmic compound, it could not be classified as a 'local anaesthetic' type. Although anti-arrhythmic compounds with direct effects on the cardiac membrane in reducing MRD usually also have local anaesthetic properties on nerve, the relative activities of various compounds on the two tissues are often very different. In the present paper we have found Org 6001 to be 1.8 times more potent than procaine on desheathed frog nerve. However, the onset of action of Org 6001 is much slower than that of procaine, and nerves exposed to concentrations of Org 6001 greater than the ED_{50} never fully recovered from the effects of the drug. Thus, at any rate at high concentrations, Org 6001 exhibited some neurotoxicity in addition to local anaesthesia. Such 'local anaesthetic' concentrations are of academic interest only, being about a hundred times greater than would be found in the plasma of treated patients.

In rabbit isolated atria Org 6001 had no effect on resting membrane potential at concentrations up to 46.8 μM. At a much lower concentration (2.9 μM) Org 6001 caused a statistically significant reduction in the maximum rate of depolarization (MRD) in association with a positive inotropic action. Even at the highest concentration used, the small depression of contractions by Org 6001 was not statistically significant. It was concluded, therefore, that Org 6001 had high direct membrane activity of class 1 type, with a negligible negative inotropic action. In common with other class 1 drugs, Org 6001 was much less effective in low K solutions than in those containing K in a concentration equal to that of rabbit plasma.

In isolated atria chronotropic responses to isoprenaline were not affected by Org 6001, and so there appeared to be no class 2 (anti-sympathetic) action on the heart. Class 3 action (prolongation of the action potential plateau) was also minor in isolated atria. Finally, by means of a simple new method for testing antagonism to the positive inotropic action of increases in calcium concentration, Org 6001 had no activity of the type exhibited by verapamil (Class 4).

The question arises whether the class 1 activity of Org 6001 is adequate to explain its anti-arrhythmic properties. There may well be types of anti-arrhythmic action other than the four classes described above. It is certain that, within the group of drugs possessing class 1 activity, subdivisions can be made on the basis of various side effects. For example, quinidine and disopyramide have atropine-like properties. Lignocaine and diphenylhydantoin have considerable action on the central nervous system. Lignocaine, and to a lesser extent propranolol also, shorten the action potential at high concentrations, especially in the Purkinje system. Whether these side effects have any relevance to their anti-arrhythmic properties is unproven. Certainly a shortening of the action potential duration would be likely to precipitate arrhythmias rather than to prevent them (Olsson, Cotoi & Varnauskas, 1971; Gavrilescu & Cotoi, 1972). What all these compounds have in common, in addition to their anti-arrhythmic effect, is the ability, in normal K solutions, to reduce MRD in the absence of any change in resting potential. On Occam's principle that hypotheses are not to be unnecessarily multiplied, the onus of proof that an extra hypothesis is necessary rests upon those who claim that the 'class 1' action of local-anaesthetic type drugs is inadequate to explain their anti-arrhythmic action.

In ventricular muscle the effects of Org 6001 on intracellular potentials were similar to those in atrial muscle; no change in resting potential, a large fall in MRD at low dosage, and a small lengthening of action potential duration. This is in contrast to the action of lignocaine, which shortens the APD in ventricular muscle (Wittig *et al.*, 1973).

However, in the ventricular conducting system Org 6001 shortened APD at all points. The effect was small in the bundle of His, but much greater in the central region in which the APD is normally much longer than elsewhere. As a result of these changes (some shortening in His, big effect in the middle, and lengthening in the muscle), the APD was made virtually uniform throughout by a concentration of 29.3 μM Org 6001. The question arises whether this shortening of APD in the conducting system is relevant to the anti-arrhythmic action of the drug. Presumably the very long APD in the distal Purkinje fibres has some functional significance, such as ensuring that ventricular muscle repolarizes first, to block any possibility of retrograde excitation of Purkinje fibres by ventricular muscle. Shortening of Purkinje APD might, therefore, in isolation be pro-arrhythmic, but less so in the presence of a simultaneous strong class 1 effect. Evidence already quoted associates short action potentials with greater probabilities of fibrillation.

On the other hand inhomogeneity is one of the factors predisposing to arrhythmias in 2- or 3-dimensional networks. Non-uniform recovery of excitability in ventricular muscle, which constitutes such a network, was demonstrated by Han & Moe

(1964) and was considered to be likely to enhance a tendency to arrhythmias (Moe, 1962). Thus the elimination of such inhomogeneity in the conducting system has been put forward as a possible reason for the anti-arrhythmic action of lignocaine (Wittig *et al.*, 1973) and propranolol (Harrison, Wittig & Wallace, 1973). However, the arguments for inhomogeneity being a predisposing factor to re-entry would not apply to a linear uni-directional conducting system.

Whether elimination of inhomogeneity in the conducting system (as opposed to the ventricular muscle) is a pro- or anti-arrhythmic effect remains to be proven. Perhaps the other effects of Org 6001 on Purkinje fibres, notably a raised electrical threshold, increased latency, reduced MRD and elimination of spontaneous pacemakers, may be of more significance to its anti-arrhythmic action.

The class 1 action of Org 6001, common to so many anti-arrhythmic drugs, was unequivocal in all tissues studied, and the drug appears to possess none of the other three classes of anti-arrhythmic action to any significant extent, though the small class 3 effect in atrial and ventricular muscle could be a contributing factor. It has virtually no depressant action on contractions in concentrations with high membrane activity. Since it is also rapidly absorbed when taken orally, has a long duration of action, and has very low general toxicity (Vargäftig *et al.*, 1975), Org 6001 obviously merits consideration for clinical trial, especially in maintenance or prophylactic therapy.

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EFFECTS OF SEVERAL INHALATION ANAESTHETICS ON THE KINETICS OF POSTSYNAPTIC CONDUCTANCE CHANGES IN MOUSE DIAPHRAGM

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1 Miniature endplate currents were recorded with extracellular electrodes in mouse diaphragms in order to measure the kinetics of the conductance change produced by acetylcholine. Miniature endplate potentials (m.e.p.ps) were recorded intracellularly in the same fibres in which the currents were being recorded.

2 The general anaesthetics, ether, halothane, chloroform and enflurane at low (anaesthetic) concentrations increased the rate of decay of miniature endplate currents (m.e.p.cs) and reduced the amplitude of m.e.p.ps in this way.

3 At high concentrations the anaesthetics caused a reduction in the amplitude of both m.e.p.cs and m.e.p.ps, and a decrease in the rate of decay of the currents. With halothane and enflurane the decay of some currents became biphasic, with a prolonged tail.

4 It was proposed that the increased rate of decay of the conductance caused by the four agents at anaesthetic concentrations is due to an increase in the fluidity of the subsynaptic membrane. Prolongation of the currents at higher concentrations may be caused by an increase in membrane dielectric constant.

5 The effectiveness of the four anaesthetics in producing a 30% decrease in the time constant of decay of m.e.p.cs was shown to be related to their oil/water partition coefficients and followed closely the relationship between anaesthetic potency and oil/water partition coefficient. It is suggested therefore that the four anaesthetics may produce anaesthesia by changing the kinetics of postsynaptic conductance changes at synapses, perhaps by increasing membrane fluidity.

Introduction

It has been proposed that some pharmacological agents may depress synaptic transmission by increasing the fluidity of postsynaptic membranes (Gage, McBurney & Van Helden, 1974; Gage, 1976). Octanol, which has been shown to increase the fluidity of nerve and muscle membranes (Grisham & Barnett, 1973), increases the rate of closing of ionic channels activated by the interaction of acetylcholine with receptors so that the duration of endplate currents is decreased (McBurney & Gage, 1972; Gage *et al.*, 1974). It was suggested that the reduced duration of endplate currents was due to an increase in membrane fluidity and that any agents which increase membrane fluidity would have a similar effect.

The inhalation anaesthetics ether, chloroform, and halothane have been shown to increase the fluidity of phospholipid membranes (Johnson & Bangham, 1969; Trudell, Hubbell & Cohen, 1973). Ether and halothane also depress neuromuscular transmission (Karis, Gissen & Nastuk, 1966; Gissen, Karis & Nastuk, 1966; Galindo, 1971). The object of these experiments was to determine whether the inhalation anaesthetics depress neuromuscular transmission by reducing the duration of ionic current generated by the interaction of acetylcholine with receptors.

Brief descriptions of similar results in toad preparations have appeared elsewhere (Gage & Hamill, 1975; Hamill & Gage, 1975).

Methods

The preparation used for the experiments was the excised mouse diaphragm. Miniature endplate potentials (m.e.p.ps) were recorded with standard intracellular microelectrode techniques. Miniature endplate currents (m.e.p.cs) were recorded extracellularly using microelectrodes filled with 1 M NaCl in agar. These electrodes generally had a resistance of 0.2–1MΩ and a tip diameter of 10–100 μm. Care was taken when manipulating electrodes at junctional regions not to press too hard against the muscle fibre as pressure on nerve terminals may cause alterations in the time course of m.e.p.cs (Katz & Miledi, 1973a). When m.e.p.ps and m.e.p.cs were recorded simultaneously, m.e.p.cs were first located by searching over endplate regions with an extracellular electrode and then an intracellular electrode was inserted into the muscle fibre where m.e.p.cs were being recorded. When this was done successfully, m.e.p.cs and m.e.p.ps could be recorded simultaneously. The intracellular electrode was positioned as close as possible to the extracellular electrode (within 100 µm).

Two methods were used to record spontaneous m.e.p.cs and m.e.p.ps which occur randomly at a low frequency. In some experiments m.e.p.cs were 'captured' by use of an on-line laboratory minicomputer (LAB 8/I, Digital Equipment) which was programmed to store and display the m.e.p.cs in a stationary position on the oscilloscope screen. These m.e.p.cs could be photographed for further analysis. In most experiments a hard-wired device which performed the same function (Neurograph N3, Transidyne General Corp) was used.

The time course of m.e.p.cs was measured from film of oscilloscope traces obtained during an experiment. The trace was magnified with a film projector and projected onto calibrated graph paper. A m.e.p.c. normally has two main phases consisting of a relatively rapid increase to a maximum amplitude (peak) followed by a slower decay. These two phases have been called (Gage & McBurney, 1975) the 'growth phase' and 'decay phase' respectively. Because the growth phase of the m.e.p.cs tends to be s-shaped, and the baseline noise obscures its early part, the 'growth time' was measured as the time for an m.e.p.c. to increase from 20 to 80% of its maximum amplitude. Using the Neurograph, it was possible to 'sweep out' individual m.e.p.cs after they had been stored and hence accurate measurements could be made of the growth time. M.e.p.cs were recorded as voltage deflections with peak amplitudes of 0.05 to 1 mV. The amplitude depends essentially on the resistance between the solution in the recording microelectrode and the grounded outside solution (Gage, 1976). Because the magnitude of this resistance is never known, currents are expressed in terms of voltage rather than amperage.

The time course of an m.e.p.c. was taken to reflect accurately the time course of the transient conductance increase produced by a quantum of acetylcholine (Gage & McBurney, 1975; Gage, 1976). Because of the variation in their time course, 20–30 m.e.p.cs were averaged for any one estimate of m.e.p.c. time course.

The anaesthetics, diethyl ether (ether); 2-chloro-1,1,2-trifluoroethyl-difluoromethyl ether (enflurane); 1,1,1-trifluoro-2 bromoethane (halothane) and chloroform were dissolved directly in the normal physiological mouse saline (Krebs solution) which was bubbled with a gas mixture of 5% CO_2 and 95% O_2, giving a pH of 7.2.

The bath temperature was controlled to within ±0.5°C. Experiments were usually done at temperatures below 30°C rather than at higher temperatures because this seemed to improve the durability of preparations. Later experiments have shown similar effects at 37°C (Hamill, Gage & Spence, 1976).

Results

As in amphibian muscle, m.e.p.cs in the mouse diaphragm had a rapid growth phase and slower decay phase (Gage & Armstrong, 1968; Gage & McBurney, 1972; 1975). The growth time (20–80%) ranged from 100 to 300 µs at 23°C. The decay was exponential as in amphibia (Takeuchi & Takeuchi, 1959; Magleby & Stevens, 1972a; Gage & McBurney, 1972). This is illustrated in Figure 1 in which the lower trace (points) show an m.e.p.c. recorded at 23°C. Inset is a graph of the logarithm of current amplitude against time from the peak and the decay is clearly exponential. The time constant of decay in this experiment was 1.4 milliseconds. It was found that m.e.p.cs in mouse diaphragm had a more rapid decay than m.e.p.cs in toad muscle (Gage & McBurney, 1972; 1975) at the same temperatures. In the upper trace is shown the m.e.p.p. generated by the current below. Its time course is slower than that of the current because of the capacity of the surface membrane of the muscle fibre.

Low concentrations of the anaesthetics

Low concentrations (see Table 1) of all four anaesthetics caused a more rapid decay of m.e.p.cs but no decrease in their amplitude. Because the integral of the current (charge displacement) was reduced, m.e.p.ps were reduced in amplitude (Gage & McBurney, 1973). These effects seen with 1 mM halothane are illustrated in Figure 2. The experiment was carried out at 20°C. A m.e.p.p. (above) and the m.e.p.c. (below) which generated it, were recorded in control solution (a). The time constant of decay of the m.e.p.c. was 1.9 milliseconds. After 15 min in 1 mM halothane (b) there was a noticeable increase in the rate at which m.e.p.cs decayed and the time course of their decay was still exponential. The time constant of decay of the m.e.p.c. in Figure 2b was reduced 37% to 1.2 ms by the halothane solution. The decreased decay time constant of the currents was associated with a reduction in m.e.p.p. amplitude (Figure 2b) as would be expected (Gage & McBurney, 1973). In this case (Figure 2) there was a 32% reduction in the amplitude of the m.e.p.p.

The decay of m.e.p.cs in the presence of low concentrations of all four anaesthetics remained exponential. This is illustrated in Figure 3 in which the logarithm of the amplitude of m.e.p.cs (recorded in

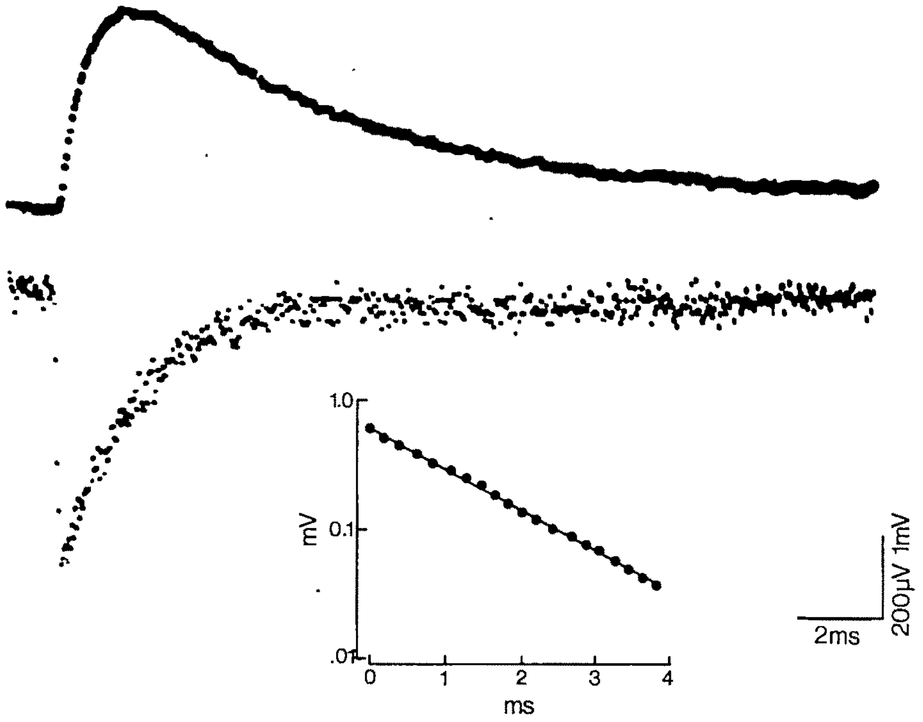

Figure 1 A miniature endplate potential (m.e.p.p.) (above) and miniature endplate current (m.e.p.c.) (below) recorded in control solution (23°C). Vertical calibration; 1 mV for the upper trace, 0.2 mV for the lower. Horizontal calibration; 2 ms. Inset is a semilogarithmic plot of current amplitude (mV) against time measured from the peak (ms) illustrating the exponential decay of the m.e.p.c.

four different experiments) has been plotted against time after the peak, in 10 mM ether, 1 mM chloroform, 1 mM enflurane and 1 mM halothane. There was no appreciable deviation from exponential decay and the time constant of decay was significantly reduced by each of the anaesthetics.

The pooled results from several experiments with low concentrations of each of the four anaesthetics are shown in Table 1. It can be seen that they all caused a significant decrease in the time constant of decay of m.e.p.cs. This effect was not accompanied by any change in the resting potential of muscle fibres and endplate potentials could still be elicited by stimulation of the phrenic nerve indicating that excitation-secretion coupling in the nerve terminals remained intact.

Higher concentrations of anaesthetics

The decrease in the time constant of decay of m.e.p.cs was found not to increase progressively with anaesthetic concentration. The changes in time course

Table 1 Reduction in the time constant of decay (τ) of miniature endplate currents caused by inhalation anaesthetics (23°C)

Anaesthetic	Concentration (mM)	τ (ms) Control	 Test	% Reduction in τ
Ether	10	1.62 ± 0.02	1.065 ± 0.02	34%
Halothane	0.2	1.68 ± 0.02	1.18 ± 0.02	29%
Chloroform	0.5	1.51 ± 0.05	0.99 ± 0.01	34%
Enflurane	0.8	1.60 ± 0.02	1.14 ± 0.03	29%

Means are shown ± s.e. mean.

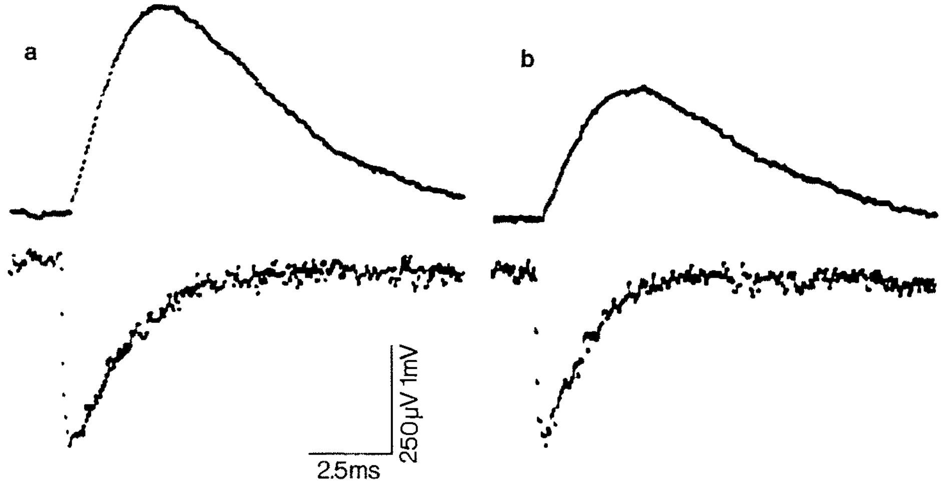

Figure 2 Halothane (1 mM) reduces the amplitude of m.e.p.ps (upper traces) by reducing the time constant of decay of m.e.p.cs (lower traces). Representative records are shown in control solution (a) and in 1 mM halothane (b). Vertical calibration; 1 mV for m.e.p.ps, 0.25 mV for m.e.p.cs. Horizontal calibration; 2.5 ms; experiments were done at 20°C.

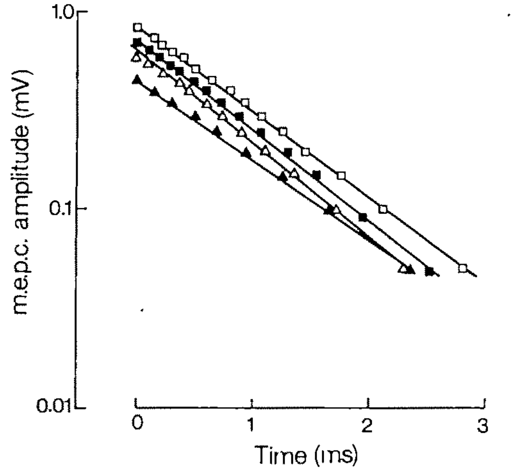

Figure 3 The amplitude of m.e.p.cs during the decay phase is plotted against time after the peak, for m.e.p.cs in 10 mM ether (□), 1 mM chloroform (■), 1 mM enflurane (△) and 1 mM halothane (▲), showing that the decay of m.e.p.cs remains exponential in the presence of the anaesthetics.

caused by higher concentrations were different for different anaesthetics and these will be described separately.

Ether. The effects of high concentrations of ether on m.e.p.cs are illustrated in Figure 4 with representative records obtained in one experiment. A m.e.p.c. in the control solution is shown in Figure 4a. In (b) (45 mM ether), there was a reduction in the amplitude of m.e.p.cs and an increase in the time constant of decay. This produced m.e.p.ps which were reduced in amplitude and had a slower time course than normal. These effects were even more pronounced in 55 mM ether (Figure 4c). Complete recovery (Figure 4d) was obtained by washing out the ether with control solution.

Averaged results obtained in four such experiments with a range of ether concentrations are shown in Figure 5 (23°C). The mean time constant of decay (τ) of m.e.p.cs in the control solution is shown as a horizontal line with ± 1 s.e. mean (broken lines). The points with vertical bars show the mean $\tau \pm 1$ s.e. mean measured from 20–30 m.e.p.cs in each of the four preparations. There is clearly a reduction in τ with ether concentrations from 2 to 25 mM. At concentrations above 40 mM, ether caused an increase in τ. However, the increases in τ were associated with a decrease in amplitude of m.e.p.cs so that no increase in amplitude of m.e.p.ps was produced by the lengthening of the current (Gage & McBurney, 1973). It was noticed that there was no significant change in the growth time of m.e.p.cs in spite of the large changes in τ.

Halothane differed from ether in two ways. Firstly, significant changes in the time constant of decay of m.e.p.cs occurred at much lower concentrations of halothane, 0.2 mM halothane producing much the same effect as 7.5 mM ether. Secondly, with higher

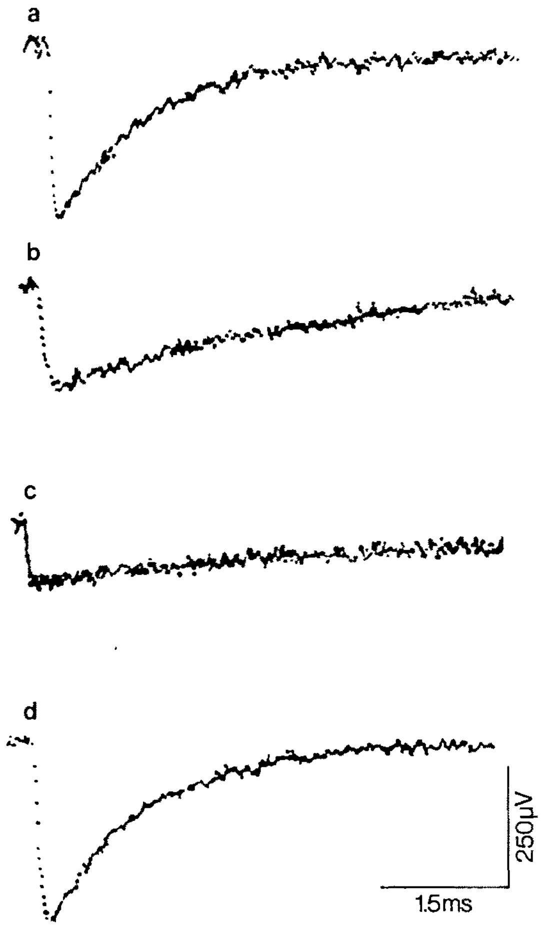

Figure 4 The effect of high concentrations of ether on m.e.p.cs. Currents are shown in control solution (a), in 45 mM ether (b), in 55 mM ether (c) and after washout of the ether with control solution (d). The figure illustrates the increase in decay time constant and reduction in amplitude of m.e.p.cs produced by high concentrations of ether. Vertical calibration; 0.25 mV. Horizontal calibration, 1.5 ms.

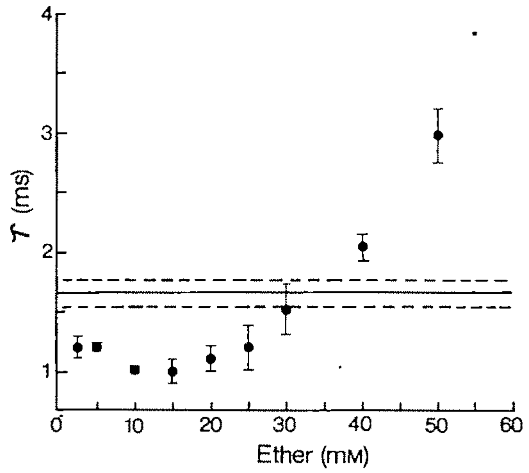

Figure 5 The effect of a wide range of ether concentrations (mM) on the time constant of decay (τ) of m.e.p.cs. The points and vertical bars show mean τ ± 1 s.e. mean in ether. The horizontal lines show the mean τ ± 1 s.e. mean in control solution. Experiments were done at 23°C.

concentrations of halothane (more than 2 mM), at any one endplate the decay of some m.e.p.cs was no longer exponential but developed a slow component so that two rates of decay could be seen. Other m.e.p.cs had a prolonged exponential decay. Biphasic m.e.p.cs were never seen with ether concentrations up to 55 mM. Ar example of one of these 'biphasic' m.e.p.cs recorded ir 20 mM halothane is shown in Figure 6b. An m.e.p.c recorded at the same site in control solution is showr for comparison in Figure 6a. Figure 6 also illustrate the reduction in amplitude of the m.e.p.p. (uppe trace). The change in time course of the m.e.p.p. (th slowing of both rising and early decay phases) is a would be expected (Gage & McBurney, 1973) fron the m.e.p.c. waveform.

The effect of a wide range of halothane concentrations on m.e.p.cs recorded in two experiment are shown in Figure 7. With concentrations abov 5 mM, measurements were made only on m.e.p.c having an exponential decay which could be describe by a single time constant. Again it can be seen tha there was a decrease in decay time constant at lowe concentrations, but an increase at higher concentrations. The effects could always be rapidly an completely reversed by washing out the halothan with normal solution.

Chloroform produced a significant reduction in τ a a concentration of 0.5 mM (Table 1) and wit

Table 2 Reduction in the time constant of decay (τ) of miniature endplate currents caused by a range of chloroform concentrations (25°C)

	Control	*Chloroform*			
Concentration (mM)	0	0.5	0.75	1	1.5
Mean (ms)	1.20	0.74	0.64	0.78	1.10
s.e. mean	0.04	0.03	0.04	0.05	0.07

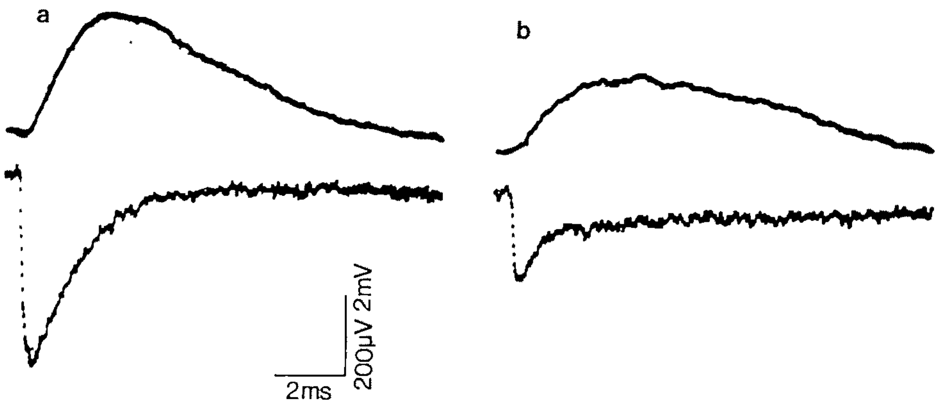

Figure 6 The effect of 20 mM halothane on m.e.p.ps (upper traces) and m.e.p.cs (lower traces). In 20 mM halothane (b), the m.e.p.c is biphasic and reduced in amplitude compared with the control (a), producing a prolonged m.e.p.p. also reduced in amplitude (b). Vertical calibration; 2 mV for upper traces, 0.2 mV for lower traces. Horizontal calibration; 2 ms.

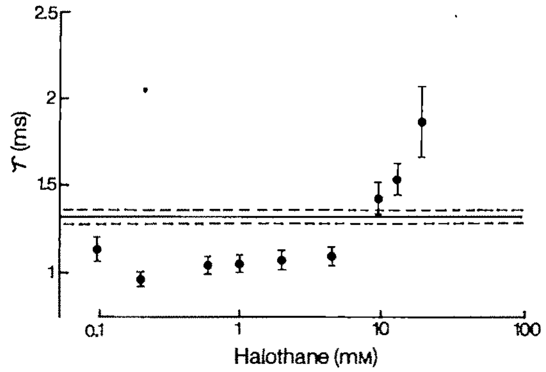

Figure 7 The effect of a range of halothane concentrations on the time constant of decay (τ) of m.e.p.cs. The points and vertical bars show mean τ±s.e. mean in halothane. The horizontal lines show the mean τ ±s.e. mean in control solution. A logarithmic horizontal axis was used to cover the wide range of concentrations. Experiments were done at 25°C.

concentrations up to 0.75 mM there was an even greater reduction in τ (Table 2). Concentrations above 20 mM produced an increase in τ and in this respect chloroform was similar to ether, although chloroform did not produce as great an increase in τ as ether. The effects of a range of chloroform concentrations from one experiment are shown in Table 2.

Enflurane was similar to halothane in that shortening of m.e.p.cs was observed at low concentrations (0.75–2 mM). Biphasic and prolonged currents were observed at higher concentrations (10 mM).

Discussion

The inhalation anaesthetics, ether, halothane, chloroform and enflurane, reduced the amplitude of m.e.p.ps at all concentrations tested. At low concentrations (Table 1), they did this by reducing the time constant of decay of m.e.p.cs. Intravenous anaesthetics also increase the rate of decay of endplate currents (Seyama & Narahashi, 1975; Adams, 1975; Quastel & Linder, 1975; T. Torda & P.W. Gage, unpublished observations). At higher concentrations, the reduction in amplitude of m.e.p.ps produced by the inhalation anaesthetics was caused by a decrease in the amplitude of m.e.p.cs which also became prolonged, or biphasic with prolonged tails. Ether, and chloroform to a lesser extent, increased the time constant of decay of m.e.p.cs. Halothane and enflurane produced both biphasic and prolonged m.e.p.cs. Previously obtained results are not inconsistent with these observations if the possible influence of concentration is taken into account. Ether has been noted to reduce the average life-time of endplate channels (Katz & Miledi, 1973b) and ether and halothane have been reported to produce lengthened and biphasic currents in voltage-clamped fibres (Quastel & Linder, 1975).

The time course of an m.e.p.c. reflects the time course of the endplate conductance change generated by a quantum of acetylcholine (Gage & McBurney, 1975; Gage, 1976). Under normal conditions the rate of decay of this conductance is controlled by the rate

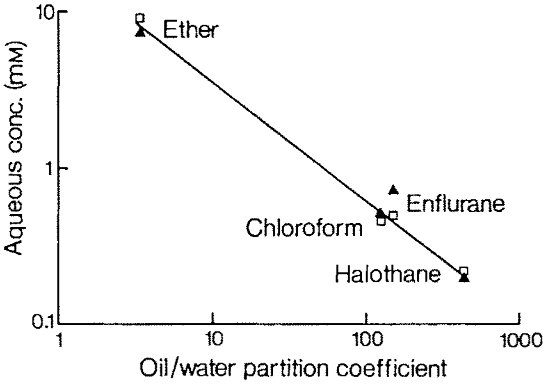

Figure 8 The aqueous concentrations (mM) of the four anaesthetics producing a 30% reduction in the time constant of decay of m.e.p.cs (triangles) are plotted against oil/water partition coefficients (taken from Secher, 1971; Dobkin *et al.*, 1971). Note the logarithmic ordinate and abscissa scales. The aqueous concentrations of the anaesthetics which produce anaesthesia (see Table 3) are plotted against partition coefficient on the same graph (squares) to show the close correlation between concentrations producing either effect.

of transition of ionic channels from open to closed conformation, as can be shown by comparing power spectra of m.e.p.cs and acetylcholine current noise (Katz & Miledi, 1972; Anderson & Stevens, 1973). Thus any increase in the rate of decay of m.e.p.cs is probably associated with an increase in the rate of this transition. (Another possibility is that an increase in the rate of decay of m.e.p.cs caused by pharmacological agents could be due to blocking of channels at a rate more rapid than the normal rate of closing). It has been proposed (Magleby & Stevens, 1972b) that the normal, rate-limiting step is a conformational change in a protein. If this is so, then low concentrations of the anaesthetics may increase the rate of this conformational change. Possible ways in which they might do this could be by interacting directly with the protein or with its environment. It is not possible at present to exclude either alternative but as ether, chloroform and halothane (Johnson & Bangham, 1969; Trudell *et al.*, 1973) have been shown to increase the fluidity of phospholipid membranes it seems not unlikely that they would also increase the fluidity of the lipid phase of postsynaptic membranes. If so, the change in time constant of decay of m.e.p.cs would be related to the concentration of the anaesthetics in membrane lipid. The good correlation which can be seen (Figure 8) between the aqueous concentrations which caused a 30% decrease in the time constant of decay of m.e.p.cs (triangles) and the oil/water partition coefficients of the anaesthetics is consistent with this hypothesis. It would appear that equal lipid concentrations of the anaesthetics have approximately the same effect on the rate of the conformational change which determines the life-time of ionic channels, as would be expected if the effect is due to an increase in membrane fluidity. However, the same correlation (Figure 8) might be obtained if the anaesthetics were acting at some hydrophobic protein site. A similar correlation between synaptic depression and hydrophobicity has been reported previously (Barker, 1974; 1975).

An increase in membrane fluidity does not explain the lengthening of currents or the biphasic m.e.p.cs caused by higher concentrations of these agents. It seems unlikely that these effects are due to inhibition of acetylcholinesterase. Anticholinesterases slow both the growth phase and decay phase of m.e.p.cs and the decay phase remains essentially exponential (Gage & McBurney, 1975) whereas the anaesthetics did not slow the growth phase of m.e.p.cs. Furthermore, halothane and enflurane gave some m.e.p.cs with a biphasic decay. Such an effect has not been reported for anticholinesterases.

Short-chain, aliphatic alcohols such as ethanol also produce an increase in the time constant of decay of m.e.p.cs (Gage, McBurney & Schneider, 1975). It was proposed (Gage *et al.*, 1975) that the effect was due to an increase in the dielectric constant of the membrane resulting from the entry of lipid-soluble substances with dielectric constants greater than 3 (the dielectric constant assumed for membrane lipid) into the postsynaptic membrane. If the rate-limiting reaction determining the decay of m.e.p.cs is accompanied by a decrease in the dipole moment of the reactant (Magleby & Stevens, 1972b; Gage & McBurney, 1975; Gage *et al.*, 1975) an increase in the dielectric constant of the environment of the reactant would slow the reaction rate (Glasstone, Laidler & Eyring, 1941). Ether and chloroform have dielectric constants greater than 3 (Weast, 1972) and may also prolong currents by increasing membrane dielectric constant. It may be that the biphasic m.e.p.cs are generated by two different populations of receptors, the prolonged phase being generated by those receptors which 'see' an increased dielectric constant in their environment and hence relax more slowly.

It is interesting that such different chemical agents as hexanol (Gage *et al.*, 1975), procaine (Maeno, 1966; Gage & Armstrong, 1968; Kordas, 1970; Maeno, Edwards & Hashimura, 1971; Deguchi & Narahashi, 1971; Adams, 1975), halothane and enflurane all produce biphasic m.e.p.cs but the origins of the effect may not be the same.

The question naturally arises as to whether the effects described here also occur at central synapses during anaesthesia. Certainly the low concentrations of anaesthetics are within the ranges of concentrations which would be found in an aqueous phase equilibrated with the blood or alveolar gas of an animal anaesthetized to a level appropriate for surgical procedures. Concentrations in the aqueous phase given in Table 3 were calculated from blood concentrations and minimum alveolar concentrations (MAC; the concentration which abolishes motor responses to a painful stimulus in 50% of a test group of animals; Eger, Brandstater, Saidman, Regen, Severinghaus & Munson, 1965) associated with different levels of anaesthesia. Partition coefficients, MACs and blood concentrations are given by Secher (1971) for ether, halothane and chloroform, and by Dobkin, Byles, Ghanooni & Valbuena (1971) for enflurane. Where both blood concentrations and MACs were available, there was found to be little difference in aqueous concentrations calculated from either value. It can be seen that the anaesthetic concentrations in Table 1 are reasonably close to the calculated aqueous concentrations for anaesthesia in Table 3. This is illustrated also in Figure 8 in which aqueous concentrations, calculated from MACs and blood concentrations producing anaesthesia (squares), are plotted against oil/water partition coefficients for comparison with the concentrations producing a 30% decrease in the time constant of decay of m.e.p.cs (triangles). Considering the wide range of partition coefficients, the correlation between concentrations of the anaesthetics which produce a 30% reduction in the time constant of decay of m.e.p.cs and concentrations which produce anaesthesia is very good.

The good correlation does not prove, of course, that the anaesthetics produce the two effects in the same way. However, the general anaesthetics do increase membrane fluidity (Johnson & Bangham, 1969; Trudell *et al.*, 1973) and it seems reasonable to suppose that this is the cause of the increased rate of decay of the postsynaptic conductance change caused by the low concentrations of the anaesthetics. If the effects of the low concentrations described here are caused by an increase in membrane fluidity rather than by interaction with acetylcholine receptors, it would not be surprising if they had a similar effect on postsynaptic conductance changes in the central nervous system. As at the neuromuscular junction, this would cause a reduction in the amplitude of postsynaptic potentials and a decreased 'sensitivity' of the subsynaptic membrane to agonists. Although the reduction in amplitude of m.e.p.ps caused by the anaesthetics was less than 40%, and would have little effect on transmission at the neuromuscular junction where there is a high safety factor, such a reduction in the amplitude of postsynaptic potentials in the central nervous system would have a significant effect on synaptic transmission.

The different effects of the anaesthetics at different concentrations suggest that they had at least two different actions, one to shorten and the other to lengthen the duration of the conductance change. Although only shortening of currents was seen with low concentrations, both actions may normally be superimposed so that there is a net shortening of currents at low concentrations and a net lengthening at higher concentrations. It should be noted that the higher concentrations producing prolonged and biphasic currents are greater than those which would cause respiratory arrest in animals.

As suggested above, the anaesthetics at higher concentrations may increase the duration of the conductance change by increasing the membrane dielectric constant. This would prolong the conductance change at amphibian and mouse neuromuscular junctions if the decay of conductance is associated with a decrease in dipole moment of the receptor (Magleby & Stevens, 1972b; Gage & McBurney, 1975; Gage *et al.*, 1975). However, if at other synapses where the receptor protein is different, the decay of the conductance is associated with an

Table 3 Aqueous concentrations of the anaesthetics calculated from blood concentrations (37°C)

Anaesthetic		*Analgesia*	*Concentration for Anaesthesia*	*Resp. arrest*
Ether	Blood (mg%)	40–50	70–140	160–180
	Aqueous (mM)	6–7	10–21 (9)	23–26
Halothane	Blood (mg%)	5	14	22
	Aqueous (mM)	0.08	0.23 (0.2)	0.36
Chloroform	Blood (mg%)	5	15	30
	Aqueous (mM)	0.15	0.46	0.9
Enflurane	Blood (mg%)		15–25	
	Aqueous (mM)		0.3–0.6 (0.5)	

Aqueous concentrations calculated from minimum alveolar concentrations are shown in brackets. Data for ether, halothane and chloroform are from Secher (1971) and for enflurane, from Dobkin, Byles, Ghanooni & Valbuena (1971).

increase rather than a decrease in the dipole moment of the receptor, an increase in dielectric constant would increase the rate of decay of the conductance. At such a synapse, the two mechanisms seen at the toad (Gage & Hamill, 1975) and mouse neuromuscular junctions, instead of having the opposite effect, would be acting in the same direction and this would lead to an even greater decrease in the time constant of decay of currents and hence in the amplitude of postsynaptic potentials. Such a possibility does not seem implausible. For example, at crustacean neuromuscular junctions, the decay of synaptic current is shortened as the postsynaptic membrane is hyperpolarized (Dudel, 1974). By analogy with the explanation given for the voltage sensitivity at cholinergic neuromuscular junctions where hyperpolarization lengthens rather than shortens currents, this could be caused by an increase rather than a decrease in the dipole moment of relaxing receptors. In agreement with this idea, it has been found in preliminary experiments at crab neuromuscular junctions that ethanol shortens synaptic currents (Adams, Hamill & Gage, unpublished observations). Such an effect would be expected (Glasstone *et al.*, 1941) if ethanol increases membrane dielectric constant (Gage *et al.*, 1975) and the relaxation of the receptor is accompanied by an increase in the dipole moment of the reactant. At such synapses an increase in dielectric constant caused by general anaesthetics would similarly produce synaptic currents of briefer duration and hence synaptic depression.

In conclusion, it has been shown that low (anaesthetic) concentrations of ether, halothane, chloroform, and enflurane depress the amplitude of m.e.p.ps by increasing the rate of decay of the conductance change produced by a quantum of acetylcholine. If they do this by increasing membrane fluidity as proposed, they should have a similar effect at synapses in the central nervous system. At higher concentrations the general anaesthetics prolong the decay phase of m.e.p.cs. If this is caused by an increase in membrane dielectric constant (Gage *et al.*, 1975), the effect of these anaesthetics on synaptic currents at other synapses will depend on the sign and magnitude of any change in dipole moment of the receptor as it relaxes from open to closed conformation. Such properties of different receptors at different synapses, as exemplified already at peripheral neuromuscular junctions, may underlie some selective actions of different anaesthetic agents in the central nervous system.

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INDOMETHACIN-INDUCED ALTERATIONS IN CORTICOSTEROID AND PROSTAGLANDIN RELEASE BY ISOLATED ADRENOCORTICAL CELLS OF THE CAT

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1 The effects of purported prostaglandin synthesis inhibitors on steroid and prostaglandin (E and F) release from trypsin-dispersed cat adrenocortical cells were investigated.

2 Low indomethacin concentrations potentiated adrenocorticotrophin (ACTH)-evoked prostaglandin and steroid release, whereas higher concentrations depressed both responses to ACTH. The steroidogenic response to exogenous prostaglandin E_2 was not markedly altered over a wide range of indomethacin concentrations.

3 Indomethacin enhanced basal steroid release but did not enhance basal prostaglandin E or F release.

4 5, 8, 11, 14-Eicosatetraynoic acid (ETA) elicited a concentration-dependent inhibition of ACTH-induced steroid release, but had little effect on prostaglandin E_2-induced steroid release. A high concentration of ETA inhibited prostaglandin E and F release.

5 These data are discussed in relation to the concept that prostaglandins provide a critical link in ACTH-induced corticosteroidogenesis.

Introduction

Prostaglandins are believed to act as mediators in a variety of cellular functions, including secretion (Horton, 1969; Flack, 1973). Although the adrenal cortex represents a secretory organ which has been studied extensively in regard to the possible roles of calcium and cyclic adenosine-3′,5′-monophosphate (AMP) as potential intermediates in the steroidogenic process (cf. Halkerston, 1975), the participation of prostaglandins in the mechanism of action of adrenocorticotrophin (ACTH) in triggering steroidogenesis is at present unresolved (Flack, 1973; Shaw & Tillson, 1974). Evidence supporting an interrelationship between prostaglandins and steroidogenesis derives, in part, from pharmacological data, which demonstrate that high concentrations of exogenous prostaglandin of the E series are capable of enhancing steroidogenesis *in vitro* (Flack, Jessup & Ramwell, 1969; Warner & Rubin, 1975). Evidence for the involvement of endogenous prostaglandins in the action of ACTH was provided by our own recent studies which showed that in cat isolated adrenocortical cells, ACTH enhances the biosynthesis of prostaglandins E and F from radiolabelled arachidonic acid (Laychock & Rubin, 1975).

Another approach to ascertaining the role of prostaglandins is to study the effects on steroid production of agents whose primary pharmacological action involves an inhibition of prostaglandin synthesis. The antirheumatic agent, indomethacin, is one of the more potent members of this group (Ferreira & Vane, 1974; Flower, 1974). This investigation is concerned with the effects of indomethacin on ACTH-induced steroid and prostaglandin release from isolated adrenocortical cells; it provides additional evidence for the concept that prostaglandins function as mediators of corticosteroidogenesis.

Methods

Cortical cell suspension

Both adrenal glands were excised from cats and the cortical cells dispersed with trypsin by the procedure described by Rubin & Warner (1975). The cells were maintained in Dulbecco's Modified Eagle's medium (GIBCO) (MEM) supplemented with 0.2% bovine serum albumin and 0.04% trypsin inhibitor when prostaglandin analysis was performed, and 0.6% bovine serum albumin and 0.1% trypsin inhibitor when optimum responsiveness to ACTH was desired for measurement of corticosteroid release.

Steroid and prostaglandin analysis

Following their dispersion, the cells, averaging 2.5×10^5 cells/ml, were incubated for 60–90 min at 37°C in the presence or absence of stimulant and/or inhibitor. The cell suspensions were then centrifuged at 6000 rev/min for 10 min at 4°C, and the supernatant assayed for corticosteroid by competitive protein binding (Jaanus, Carchman & Rubin, 1972).

Prostaglandin analysis of the supernatant was carried out by radioimmunoassay (RIA) using antiserum to prostaglandin $F_{2\alpha}$ produced in rabbits against prostaglandin $F_{2\alpha}$-bovine serum albumin conjugates (Caldwell, Burstein, Brock & Speroff, 1971). Prostaglandin F determinations were made by direct RIA of 300 μl aliquots of cell incubation medium. For the prostaglandin E determination, 3 ml of supernatant was pooled and acidified to pH 3 with 0.5N HCl and processed through XAD-2 columns, according to the method of Keirse & Turnbull (1973). The ethanol eluate was evaporated to dryness under N_2 at 50°C, resuspended in Na-phosphate buffer (pH 7.4), and subjected to reduction using sodium borohydride (0.02%), which reduces prostaglandins of the E series to prostaglandin F compounds (Levine, 1973). The prostaglandin F equivalents were determined by RIA using anti-prostaglandin $F_{2\alpha}$ antibody. The bound and free tritium labelled prostaglandin F generated during the RIA were separated by nitrocellulose filtration; the filters were dissolved in Bray's solution and counted by liquid scintillation spectrometry.

Since the details of the RIA and antibody characterization are being prepared for a subsequent publication, the prostaglandins in this study will be designated only as prostaglandin F or E equivalents, although differential assays using antisera to prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ suggest a predominance of prostaglandins E_2 and $F_{2\alpha}$ in the cell incubation medium. Steroid and prostaglandin values were expressed either in ng/2.5×10^5 cells (ng/ml) or as per cent increase relative to control cells.

Drugs and reagents

The following were used: trypsin and lima bean inhibitor (Worthington); bovine serum albumin (fatty acid free) (Sigma); [^{3}H]-corticosterone (42 Ci/mM); [^{3}H]-prostaglandin $F_{2\alpha}$ (175 Ci/mM) (New England Nuclear); non-radiolabelled prostaglandins (Upjohn). β1–24-Adrenocorticotrophin (ACTH) (Ciba); indomethacin (Merck, Sharp & Dohme) and 5,8,11,14-eicosatetraynoic acid (RO-3–1428) (Hoffman-LaRoche) were dissolved in 95% v/v ethanol and added to the final incubation volume (1 ml) in 10 μl aliquots; all other experimental samples received 10 μl ethanol. Concentrations are expressed as mol/l (M).

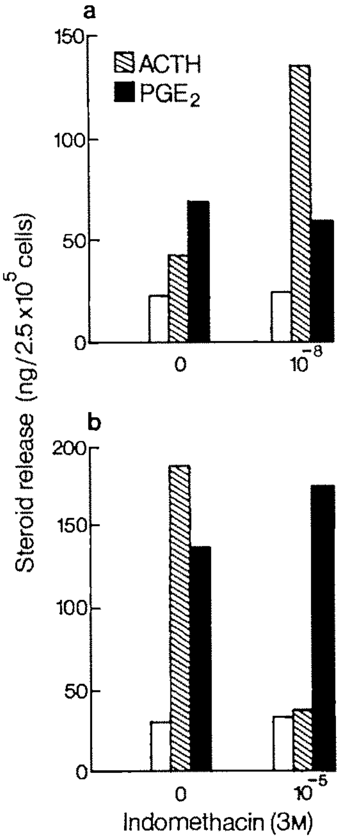

Figure 1 Comparison of the effects of indomethacin on adrenocorticotrophin (ACTH) and prostaglandin E_2 (PGE_2)-induced steroid release from isolated adrenocortical cells. Each vertical column represents the total amount of hormone released by equal number of cells during a 90 min incubation period in the presence or absence of indomethacin plus: (a) ACTH (25 μu) or prostaglandin E_2 (2×10^{-4} M), (b) ACTH (125 μu) or prostaglandin E_2 (3×10^{-5} M). The open columns show basal steroid release from unstimulated cells; hatched columns show effects of ACTH; solid columns show effects of prostaglandin E_2. These results were obtained from 2 different preparations.

Results

Effects of indomethacin on ACTH-induced steroid production

Strikingly low concentrations of indomethacin caused marked effects on ACTH-induced steroidogenesis in isolated cortical cells. Results from a single experiment using a low ACTH concentration (25 μu/ml) are depicted in Figure 1a; they show a marked facilitation of steroid release in the presence of 0.03 μM

(3×10^{-8} M) indomethacin. More detailed analysis of this potentiation using near maximal ACTH concentrations (75–250 μu) demonstrated that 3 nM (3×10^{-9}M) indomethacin augmented ACTH-induced steroid release almost two-fold (Figure 2a). This facilitatory action of the drug was transformed into an inhibitory action as its concentration was increased. ACTH-evoked steroid release in the presence of indomethacin (0.3 μM; 3×10^{-7} M) was less than in its absence (Figure 2a) and in the presence of 30 μM (3×10^{-5} M) indomethacin, a greater inhibition was detected (Figure 1b and 2a). Despite the fact that low and high concentrations of indomethacin caused considerable alterations in the steroidogenic response to ACTH, these same indomethacin concentrations did not elicit similar alterations in the steroidogenic response to exogenous prostaglandin E_2 (Figure 1).

Indomethacin action on basal steroid levels

Indomethacin in the concentration range of 3 nM to 30 μM (3×10^{-9} to 3×10^{-5} M) was capable of augmenting basal steroid release (Table 1). The stimulant effect was small and inconsistent at the lowest concentration tested (3 nM; 3×10^{-9} M), but increased approximately two-fold with 0.3 μM (3×10^{-7}) and 30 μM (3×10^{-5} M) indomethacin, respectively. This facilitatory action of indomethacin was not demonstrable to the same degree in every experiment, but was generally manifest in those preparations showing a greater responsiveness to ACTH.

Effects of indomethacin on prostaglandin release

Steroidogenic concentrations of ACTH elicited dose-related increases in release of prostaglandins E and F (Table 2–expt. 1). An indomethacin concentration (3nM; 3×10^{-9} M) that facilitated ACTH-induced steroid release, likewise potentiated the effects of ACTH on prostaglandin F and E release (Table 2–expt. 2); in the same experiment this facilitatory effect of indomethacin on prostaglandin release was converted to inhibition by a high concentration of indomethacin

Table 1 The effect of indomethacin on basal corticosteroid and prostaglandin (PG) release

Indomethacin (3 M)	*Steroid (% of basal)*	*PGF (% of basal)*	*PGE (% of basal)*
10^{-9}	113 ± 8 (4)	—	—
10^{-7}	215 ± 65 (5)	78 ± 23 (4)	75 ± 14 (3)
10^{-5}	174 ± 35 (5)	87 ± 20 (6)	93 ± 35 (3)

Mean values (± s.e. mean) are expressed as % increase over values obtained in the absence of drug. Number of observations are indicated by number in parentheses; each value was obtained from a different preparation.

Table 2 Prostaglandin release from unstimulated cortical cells, and from cells exposed to ACTH with or without indomethacin

Expt.	*ACTH (μu)*	*Indomethacin (3 M)*	*PGF (pg/2.5 × 10^5 cells)*	*PGE (pg/2.5 × 10^5 cells)*
1	—	—	270	1515
	25	—	301	2201
	250	—	652	2737
	425	—	960	7426
2	—	—	179	1300
	25	—	165	1363
	25	10^{-9}	213	2645
	25	10^{-5}	79	828
3	—	—	107	633
	250	—	230	1257
	250	10^{-7}	55	524
4	—	—	72	< 6
	250	—	182	873
	250	10^{-7}*	< 6	< 6

Values for each experiment were obtained from medium incubating equal numbers of cells for 1 h at 37°C.
* Indomethacin present for 1 h prior to addition of ACTH and then removed from the medium.

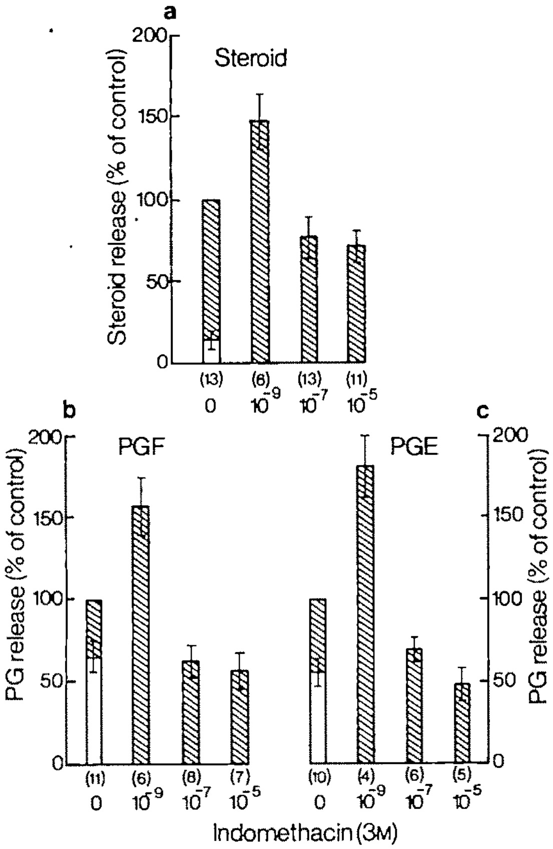

Figure 2 The effects of indomethacin on adrenocorticotrophin (ACTH)-induced steroid and prostaglandin release. Prostaglandin (PGE and PGF) and steroid determinations were made on aliquots of incubation medium following incubations of 60 and 90 min, respectively. Each vertical column represents the mean response to ACTH (±s.e.) elicited in the presence of a given indomethacin concentration; the values are expressed as a percent of the response to ACTH in the absence of drug. Mean values (±s.e.) obtained in the absence of both ACTH and indomethacin are also depicted (open columns) to illustrate the stimulant effects of ACTH in the absence of indomethacin. The number of experiments is indicated by the figure in parentheses under each column.

(30 µM; 3 x 10^{-5} M) (Table 2–expt. 2). Experiment 3 of Table 2 illustrates the potent inhibitory action of indomethacin even in the concentration of 0.3 µM (3 x 10^{-7}M). The irreversible nature of this inhibition is shown by the finding that after a 1 h exposure to indomethacin, cells reincubated in an inhibitor-free medium were unable to release measurable amounts of prostaglandin E or F in response to ACTH (Table 2–expt. 4).

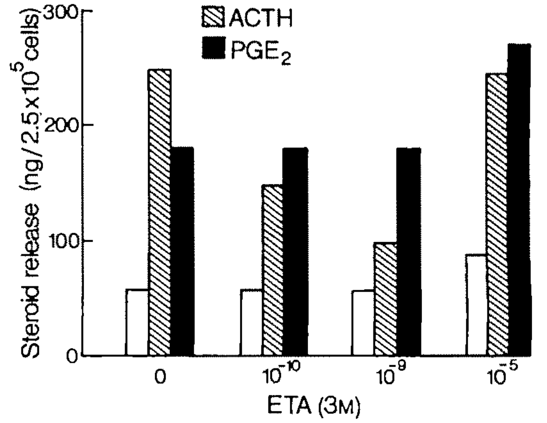

Figure 3 Comparison of the effects of 5,8,11,14-eicosatetraynoic acid (ETA) on adrenocorticotrophin (ACTH)- and prostaglandin E_2 (PGE_2)-evoked steroid release. The columns represent the total amount of steroid released by a single preparation during a 90 min incubation period with varying indomethacin concentrations, in the presence of either ACTH (50 µu, hatched columns) or prostaglandin E_2 (2 x 10^{-4}M, solid columns), or in their absence (open columns).

Figure 2 summarizes the results of all experiments, including those presented in Table 2. Indomethacin at a concentration of 3 nM (3 x 10^{-9} M) potentiated ACTH-evoked release of prostaglandins by almost two-fold. Higher indomethacin concentrations (0.3 µM and 30 µM; 3 x 10^{-7} and 3 x 10^{-5} M) depressed ACTH-evoked prostaglandin E and F values to below basal levels; with these same indomethacin concentrations, ACTH still increased steroid release by six-fold or more above basal levels (Figure 2a).

Despite the fact that indomethacin in the concentrations of 0.3 µM and 30 µM (3 x 10^{-7} and 3 x 10^{-5}M) enhanced basal steroid release, these same concentrations were unable to elicit a consistent or significant change in basal prostaglandin E or F release (Table 1).

Effects of 5,8,11,14-eicosatetraynoic acid

Although this study was primarily concerned with the action of indomethacin, several experiments were also carried out with 5,8,11,14-eicosatetraynoic acid (ETA). ETA in concentrations as low as 0.3 nM (3 x 10^{-10}M) impaired the steroidogenic response to ACTH (Figure 3). The inhibition was concentration-dependent over the range of 0.3 nM to 0.3 µM (3 x 10^{-10} to 3 x 10^{-7} M). However, at the highest ETA concentration tested (30 µM; 3 x 10^{-5} M), the inhibitory action was reversed (Figure 3). The stimulant action of exogenous prostaglandin E was not markedly affected by an ETA concentration as high as 0.3 μM (3×10^{-7} M), but its action, like that of ACTH, was potentiated by 30 μM (3×10^{-5} M) ETA (Figure 3). Basal steroid release was increased by ETA. In concentrations of 0.3 μM and 30 μM (3×10^{-7} and 3×10^{-5} M), ETA augmented basal steroid release by 121 ± 7% and 155 ± 7%, respectively (three experiments each).

In two separate experiments, ACTH-facilitated prostaglandin F release was reduced by ETA (30 μM; 3×10^{-5}M), from 1660 and 92 pg to 578 and 37 pg, respectively. This depression of the ACTH response produced by ETA was of a sufficient magnitude to cause prostaglandin E and F release to fall below basal levels. In a third experiment where ETA (30 μM; 3×10^{-5}M) failed to depress ACTH-evoked prostaglandin F release from a value of 447 pg, it decreased ACTH-induced prostaglandin E release from 1089 pg to below measurable levels.

Discussion

In assessing the potential significance of cyclic AMP in a particular physiological function, Sutherland and his associates presented certain criteria which had to be considered (cf. Robison, Butcher & Sutherland, 1971). With these criteria in mind, we have been investigating the postulate that prostaglandins provide an important link in the steroidogenic action of ACTH on isolated adrenocortical cells of cats. Previous findings show that exogenous prostaglandin E_2 is capable of augmenting steroidogenesis, and that this stimulant action resembles in several ways the action of ACTH (Warner & Rubin, 1975). In addition, ACTH enhances the conversion of radiolabelled prostaglandin precursor, arachidonic acid, into prostaglandin E and F (Laychock & Rubin, 1975). The present study provides further support for this postulate by demonstrating: (a) the presence of endogenous prostaglandins (E and F) in cat cortical cells by radioimmunoassay, (b) that ACTH enhances their release in a concentration-dependent manner and, (c) that indomethacin and ETA, agents that alter prostaglandin synthesis, cause profound changes in ACTH-evoked steroid release.

The ability of indomethacin in low concentrations to produce a parallel potentiation of steroid and prostaglandin release provides strong support for the concept that prostaglandins play a key physiological role in ACTH-induced steroidogenesis. The unexpected finding that indomethacin, a prototype inhibitor of prostaglandin synthesis, enhances release in low concentrations may relate to an ability to impair the catabolism of endogenous prostaglandins. For example, prostaglandin dehydrogenase, which catalyses the conversion of prostaglandin to a keto derivative, appears susceptible to inhibition by indomethacin (cf. Flower, 1974). Although another action not related to prostaglandin metabolism, such as inhibition of phosphodiesterase (Flores & Sharp, 1972), could account for the facilitatory action of indomethacin on ACTH-evoked steroid release, the fact that the steroidogenic action of exogenous prostaglandin E_2 was not greatly altered by a low indomethacin concentration is in harmony with the notion that indomethacin is indeed exerting its principal action on steroid release through an alteration in endogenous prostaglandin levels.

At higher indomethacin concentrations, a functional relationship between prostaglandin and steroid release was less clear. Indomethacin caused an irreversible inhibition of ACTH-induced prostaglandin E and F release, accompanied by a more modest impairment of ACTH-evoked steroid release; thus, steroid release was still augmented approximately sixfold despite prostaglandin release being at or near basal levels. This finding suggests that enhanced activity of the prostaglandin system may not be a *sine qua non* for the steroidogenic action of ACTH. However, these experiments are to be interpreted with caution since our conclusions regarding prostaglandin metabolism must be considered from the perspective that a measurement of prostaglandin release is being equated with cellular synthesis and turnover. The validity of this assumption is based upon the fact that increased prostaglandin release is a consequence of an activation of biosynthesis rather than secretion from a preformed store (Ramwell & Rabinowitz, 1972). This assumption seems justified, since in the few experiments in which we have determined intracellular prostaglandin levels by analysis of control and stimulated adrenocortical cells, their values were only 10–20% of those obtained from analysis of the incubation medium (Laychock & Rubin, unpublished observations).

The ability of indomethacin to enhance basal steroid release is also potentially an important finding, since if this increase were the result of an action on prostaglandin synthesis, then it would imply that an alteration in prostaglandin metabolism is capable of triggering steroid release in the absence of the primary stimulus, ACTH. Although this still may be the case, we were unable to detect any consistent or marked changes in basal prostaglandin E or F release caused by indomethacin.

ETA, an acetylenic analogue of arachidonic acid, inhibits the latter's conversion to prostaglandin E and F (Ahern & Downing, 1970); and in the present investigation depressed ACTH-evoked prostaglandin E and F release from cortical cells. The use of this prostaglandin analogue provided further evidence for a physiological role for prostaglandins in the regulation of steroidogenesis by causing, even in very low concentrations, a dose-dependent inhibition of ACTH-induced steroid release. The reversal of the inhibition seen with a high ETA concentration cannot be explained at present; it may be another consequence of altered prostaglandin metabolism or it may represent an additional action of the drug.

The fact that ETA did not, like indomethacin, potentiate ACTH-induced release in low concentrations, does not necessarily detract from the notion that these two agents exert their primary effects on steroid release through actions on prostaglandin metabolism. There is general agreement that indomethacin and ETA exert their actions at different sites in the biosynthetic pathway (Lands, LeTellier, Rome & Vanderhoek, 1972; Flower, 1974); the particular modification of prostaglandin synthesis elicited by each inhibitor could result in variable effects on the physiological response.

In conclusion, we feel that ample evidence now exists which is compatible with the idea that prostaglandins play some physiological role in the regulation of adrenal steroidogenesis. The extent and manner in which a particular prostaglandin is directly involved cannot be ascertained at present. Only prostaglandins E and F were analyzed in this study; and in general they were altered to a similar extent. It is entirely possible that other prostaglandins, as for example, prostaglandins A and B as well as the more labile endoperoxide intermediates, may also be involved in the action of ACTH; one or more may be facilitatory and others inhibitory. In any event, the accumulating evidence provides encouragement for the continuation of these investigations to assess more precisely the physiological significance of prostaglandins in adrenal steroidogenesis.

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ONTOGENESIS OF ENZYME SYSTEMS DEAMINATING DIFFERENT MONOAMINES

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1 A detailed investigation into the postnatal development of the activity of the enzyme monoamine oxidase (MAO) in the rat and domestic pig was carried out.

2 MAO activity was measured in littermate male rats aged between 3 and 122 days belonging to six breeding colonies. The tissues studied were three brain regions in which monoamines may play a role in neuronal transmission (septum, hypothalamus, corpus striatum) and, for comparison, in the cerebellum. Liver, heart and adrenal glands were the peripheral organs studied. The following substrates were used to measure MAO activity in each tissue homogenate: kynuramine, tyramine, dopamine, tryptamine and 5-hydroxytryptamine (5-HT).

3 MAO activity towards kynuramine, tyramine and dopamine increased after birth in all brain regions and also in the liver, to reach maximal values between days 40 and 80. In the heart and the adrenal glands enzyme activity remained low up to 30–40 days and then increased steeply. This was the case in all litters examined.

4 All tissues deaminated more tyramine than dopamine. In the liver, the ratio of the quantities of tyramine deaminated/dopamine deaminated was approx. 2 at all ages. In the homogenates of whole brains (including or excluding the hypothalamus and striatum) this ratio was also 2 at all ages. In contrast in the isolated striatum and hypothalamus it was first much higher and reached a value of 2 only at an age of about 20 days. This may indicate an independent development of a dopamine and a tyramine deaminating enzyme system in discrete brain regions. It was suggested, that the low ability to deaminate dopamine in discrete brain regions may be due to the local presence of an enzyme inhibitor which becomes too diluted to be active in homogenates of whole brain.

5 Deamination of tryptamine in the striatum decreased between day 5 and 20 in 3 out of 4 colonies tested. There was a large fall in the deamination of 5-HT in all tissues of one group of rats, but in another 4 groups the tissues of the 5 day old rats deaminated smaller amounts of 5-HT than those of the older rats.

6 Purified hypothalamic mitochondria from 40 day old rats deaminated more tyramine and dopamine but not tryptamine per mg protein than those from 5 day old rats.

7 In the domestic pig there was a significant rise in the values in hippocampal MAO activity towards dopamine and tyramine from the foetus (55 day gestation) to the 1 week old piglet. A further steady rise up to week 6 was indicated, but this rise was not statistically significant. The difference between rat and pig probably reflected the much higher degree of maturity of the latter at birth.

8 In the hippocampus of the pig the ratio between the amount of tyramine deaminated/dopamine deaminated decreased from > 10 (foetus) to 4.8 in the 6 week old pig and 2 in the adult.

Introduction

Changes in the activity of the enzyme monoamine oxidase (MAO; E.C. 1.4.3.4.) during the postnatal development of rats have been reported on several occasions. These studies led to the general conclusion that the enzyme's activity increases with age (see e.g. Novick, 1961; Karki, Kuntzman & Brodie, 1962; Bennett & Giarman, 1965; Vaccari, Maura, Marchi & Cugurra, 1972; Youdim, Holzbauer & Woods, 1974). In most of these previous studies only a single substrate has been used or only one tissue has been investigated.

In the present work a more detailed investigation has been conducted. The enzyme activity was measured in four discrete brain regions and in three peripheral organs, five different substrates being used for most samples. Litter-mate male rats from five breeding colonies aged between 5 and 80 days were used. The work was designed particularly to investigate whether the postulated multiple forms of MAO develop at the same rate during maturation, and whether any differences exist in the development of deamination of different monoamines by various organs. Some of the results have been presented to the British Pharmacological Society (Blatchford, Holzbauer & Youdim, 1975).

Methods

Rats

The experiments were carried out on litter-mate male rats from different strains and breeding colonies. All rats were weaned at day 21 and fed on a diet of Oxoid 41B (water *ad lib*). Each experimental group consisted of the male rats of three to six litters and each age group (five to six rats) included at least one rat from each litter. The average body weights for the different groups at various ages are listed in Table 1.

The rats were killed by rapid decapitation. The tissues tested for MAO activity were three brain regions rich in monoamines (hypothalamus, corpus striatum and septum) and for comparison either the posterior telencephalon or the cerebellum. The peripheral organs examined were the heart, liver and adrenal glands. The tissues were dissected out as quickly as possible and stored at −18°C. To prevent desiccation a small quantity of buffer solution was added. On the day of the enzyme assay they were homogenized in ice cold 0.1 M sodium phosphate buffer (pH 7.4) with a Polytron homogenizer. The volume of buffer used was 1 ml for the septum, striatum, hypothalamus and adrenal glands; for the cerebellum, the atria of the heart and the piece of liver (dissected from the frontal lobe) 2–10 ml was used depending on the size of the tissue.

Domestic pigs

The hippocampus and adrenal glands from two litters of pigs (breed: Large White) became available and the MAO activity in these tissues was estimated with dopamine, tyramine and tryptamine as substrates. Piglets were killed by an intracardiac injection of sodium pentobarbitone on the day of birth and 1, 2, 4 and 6 weeks of age. Each age group consisted of males and females from each litter. The MAO activity in the left and right adrenal gland and in the two halves of the hippocampus of each animal was measured separately where possible.

Enzyme assay

The MAO activity in each individual homogenate of the rat tissues was measured towards the following substrates: kynuramine, tyramine, dopamine, tryptamine and 5-hydroxytryptamine (5-HT). Enzyme activity towards kynuramine was assessed by a fluorimetric method (Kraml, 1965) as described previously (Holzbauer & Youdim, 1973). For the other amines a radiochemical method similar to that of Robinson, Lovenberg, Keiser & Sjoerdsma (1968) was used (for details see, Davis, Holzbauer, Sharman & Youdim, 1975). The same methods were used for the tissues of the piglets. The protein content of the tissue homogenates was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). Enzyme activity is expressed as nmol amine deaminated per mg protein per minute.

Thermal inactivation of the enzyme was studied by incubating the tissue homogenates (in phosphate buffer pH 7.4, 0.05 M) at 48°C. Samples for enzyme assay were taken at intervals of 20 minutes.

To study the subcellular distribution of MAO activity in the hypothalami of male Porton rats, ten hypothalami of five day old rats were pooled to form sample 1 and four hypothalami of 40 day old rats were pooled to form sample 2. The tissues were homogenized in 0.32 M sucrose. A P_1 'nuclear fraction' was obtained after centrifugation at 900 *g* for 5 minutes. The supernatant was centrifuged for 30 min at 12,000 *g*. The 'crude mitochondrial fraction' thus obtained was layered over a continuous sucrose gradient (1.4–2.0 M) and centrifuged at 100,000 *g*, for 1 h to obtained a 'true mitochondrial fraction' (Holzbauer, Bull, Youdim, Wooding & Godden, 1973).

Results

In a preliminary experiment on a group of Wistar rats (Babraham colony) kynuramine was the only substrate against which MAO activity was measured. The rats were killed when 3, 5, 9, 20, 35 and 122 days old. The tissues studied were the liver, the apex of the heart and the following brain regions: septum, striatum, hypothalamus and the posterior portion of the telencephalon including part of the hippocampus and several nuclei of the amygdaloid complex. The results obtained on the hypothalamus, posterior telencephalon, heart and liver (expressed in percent of the values on day 3) are shown in Figure 1. The homogenates of the whole brain tissues showed a rise in MAO activity with age and maximal values were seen in the 80 day old rats. This was also the case for the septa and striata (not shown on the graph). The highest absolute values (enzyme activity per mg protein per min) in the brain were found in the hypothalamus. The activity in the liver on day 3 was 3.5 times higher than in the hypothalamus; on days 35 and 122 it was 1.4 times higher. In the liver the values reached a plateau in the beginning of the second month. The heart showed low enzyme activity up to

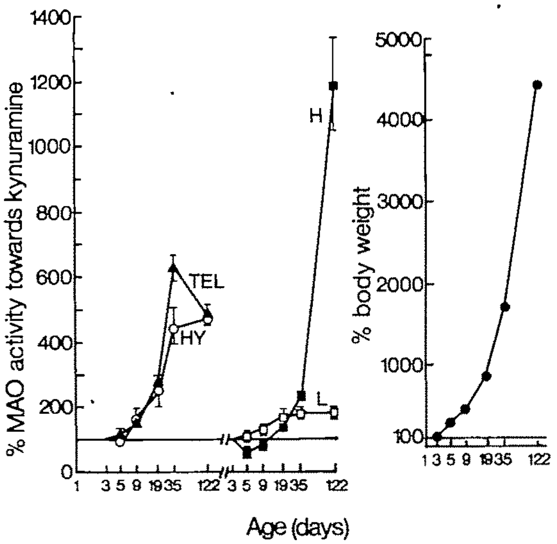

Figure 1 Relative increase in monoamine oxidase (MAO) activity (day 3 = 100%) towards kynuramine (calculated per mg protein min^{-1}) in the hypothalamus (HY, ○), posterior telencephalon (TEL, ▲), heart (H, ■) and liver (L, □) in litter-mate male rats aged 3–122 days (Babraham colony). Mean values are given; vertical lines show s.e. mean; $n = 5$. Mean body weights (●). (Preliminary experiment on a group of Wistar rats, Babraham colony).

day 35 when it amounted to only about 10% of that in the liver. Thereafter it rose steeply and in the 4 months old rat it was about 50% of that in the liver.

I. Substrate differences in the development of monoamine oxidase activity with age

Experiments were carried out on six groups of rats aged between 5 and 80 days. In each tissue homogenate MAO activity was tested against several substrates. The results are presented in Figures 2–6. In all figures the mean values obtained for each tissue at different ages are joined by straight lines irrespective of the length of the interval. These lines must not be interpreted as the exact time course at which MAO activity changes with age. The different breeding colonies are referred to as groups 1–6 (see Table 1).

(a) *Kynuramine*

Group 1. The rats used in this experiment were 5, 10, 20, 40 or 80 days old. The brain regions analysed were the hypothalamus, septum and striatum and for comparison the cerebellum. In addition the liver, the atria of the heart and the adrenal glands were studied. The results obtained with kynuramine as substrate are shown in Figure 2. Enzyme activity increased in all tissues with age. In the brain tissues the highest values were found in the 40 day old rats, in the liver in the 20 day old rats.

Group 2. MAO activity towards kynuramine was also measured in the 5 and 40 day old rats of group 2. The rise in activity with age was 80% in the whole brain homogenate, 145% in the adrenal glands and 325% in the heart. No significant rise was seen in the liver homogenate.

(b) *Tyramine.* The results obtained with tyramine as substrate in groups 1–6 are summarized in Figure 3. They are expressed as nmol tyramine deaminated mg^{-1} protein min^{-1}. The results obtained for the septum in the different groups of rats and at the various ages were very similar to those obtained for the striatum and have been omitted from the graph for the sake of clarity. The standard errors have similarly been omitted for the results obtained with the cerebellum of the rats of group 2.

Group 1. (Figure 3). In all brain regions examined and in the livers the highest enzyme activity towards

Table 1 Experimental animals

				Age (days):						
				5	*8*	*10*	*20*	*30*	*40*	*80*
Rat strain		*Colony*	*Month of birth*	*Mean body weight (g)*						
Wistar rats	Group 1	Babraham	March–May 1974	10.6	—	13.8	30.6	—	98.0	276
	Group 2	Babraham	May–June 1974	11.6	—	—	—	—	140.8	—
	Group 3	Tuck	October 1974	9.9	—	—	32.5	—	—	—
	Group 4	Tuck	March 1975	9.2	11.9	17.5	34.1	60.8	—	—
	Group 5	Porton (Babraham)	November 1974	9.6	—	—	61.0	—	—	—
Hooded rats	Group 6	Babraham	December 1974	10.4	—	—	—	76.6	—	—

Six litters of Wistar rats (Babraham colony) born in August 1973 were used in a preliminary experiment (see Figure 1). The group numbers are the same as those used in Figures 2–9. The rats belonging to the Tuck colonies were born in Babraham from mothers purchased already pregnant from Tuck.

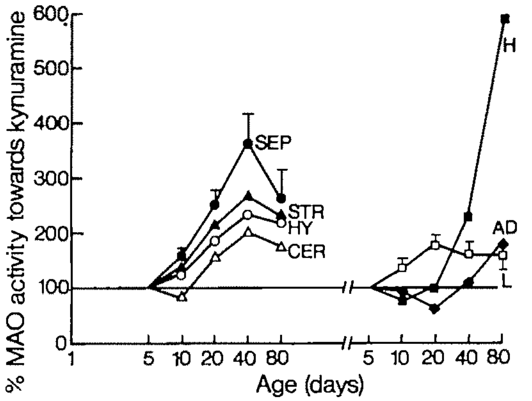

Figure 2 Relative increase in monoamine oxidase (MAO) activity (day 5 = 100%) towards kynuramine in four brain regions, heart, liver and adrenal glands. Group 1 (see Table 1), same tissue homogenates as used for the assays with tyramine, dopamine, tryptamine and 5-hydroxytryptamine in Figures 3–6, group 1. Septum (SEP, ●); striatum (STR, ▲); hypothalamus (HY, ○); cerebellum (CER, △); liver (L, □); heart (H, ■) and adrenal glands (AD, ◆) in littermate male rats aged 5–80 days; mean values are given. For the sake of clarity standard errors (vertical lines) are only given for septum and liver. $n = 4–5$. For most of the other estimates the coefficients of variation were smaller than 20%.

tyramine was seen in the 40 day old rats. In the heart and adrenal homogenates activity was low up to day 20. In both tissues there was still a considerable rise between days 40 and 80. Highest activity per mg protein in the brain was in the hypothalamus.

Group 2. (Figure 3). The rats of group 2 were killed either 5 or 40 days after birth and the whole brain, the atria of the heart, the liver and the adrenal glands were used. All tissue homogenates of the 40 day old rats deaminated more tyramine than those of the 5 day old rats. In the whole brain extracts the difference was 30%, in the liver 100%, in the heart 750% and in the adrenal glands 1000%.

Group 3. (Figure 3). The rats used in group 3 were either 5 or 20 day old. As in group 1, considerably more tyramine was metabolized by the hypothalami, striata and livers of the 20 day old rats than of the 5 day old ones. The cerebellum showed again the lowest activity of all brain regions tested.

Group 4. (Figure 3). The rats of this group were killed 3, 8, 10, 20 and 30 days after birth and only the striata were analysed. There was a threefold rise in tyramine deamination between days 5 and 20 and no further increase by day 30.

Group 5. (Figure 3). The rats of this group were killed 5 or 20 days after birth and the tissues examined were the hypothalami, the striata and the remainder of the brain including the cerebellum and medulla oblongata, and the liver. As in the other groups of rats there was a considerable rise with age in teh deamination of tyramine in all tissues studied.

Group 6. ·(Figure 3). This group consisted of hooded rats killed 5 or 30 days after birth. With tyramine as substrate only the hypothalami, the remainder of the brain as for group 5 and the livers were tested. Again all tissues showed an increase with age in their ability to deaminate tyramine.

Thus, in all groups there was a significant rise, with age, in the deamination of tyramine by all tissues studied. There were differences in the specific activity of the enzyme (nmol tyramine deaminated mg^{-1} protein min^{-1}). The MAO activity in the hypothalami of the 5 day old Porton rats (group 5) was nearly twice that of the 5 day old rats of group 1. The hypothalami of groups 3 and 5 on day 20 also exhibited a higher MAO activity per mg protein than those of group 1. The highest activity in the livers was seen in the 20 day old Porton rats.

(c) *Dopamine* The results obtained with dopamine as substrate are shown in Figure 4. All the homogenates studied deaminated less dopamine than tyramine (compare Figure 3). In many individual samples of homogenates of the four brain regions no dopamine deamination could be detected in rats aged 5 or 10 days. In the 20 day old rats of group 3 the homogenates of the striata showed no dopamine deamination, however the hypothalami had developed measurable activity. In contrast, the homogenates of whole brain of the 5 day old rats of group 2 and of group 6 (Hooded rats) deaminated considerable quantities of dopamine and there was no increase with age. No dopamine deamination was detected in the heart and adrenal homogenates of the 5, 10 and 20 day old rats. By day 40 (groups 1 and 2) and 80 (group 1) about half as much dopamine as tyramine was deaminated by the hearts. The livers deaminated dopamine about half as efficiently as tyramine at all ages. In the hypothalami of all groups tested the increase with age in dopamine metabolism was much steeper than the increase in tyramine metabolism. This was not the case for the livers.

(d) *Tryptamine.* With tryptamine as substrate, a fall with age in the MAO activity of the brain regions studied became apparent (Figure 5). Because a decrease in MAO activity with age was an unexpected finding, special care was taken to test the validity of the enzyme assay method. Enzyme kinetic studies (on homogenates of hypothalami of 5 and 40 day old rats) using tryptamine as substrate showed however that deamination of this amine followed first order kinetics.

There were differences between the groups of rats studied, in the rate at which this decrease with age occurred and also in the absolute quantities of

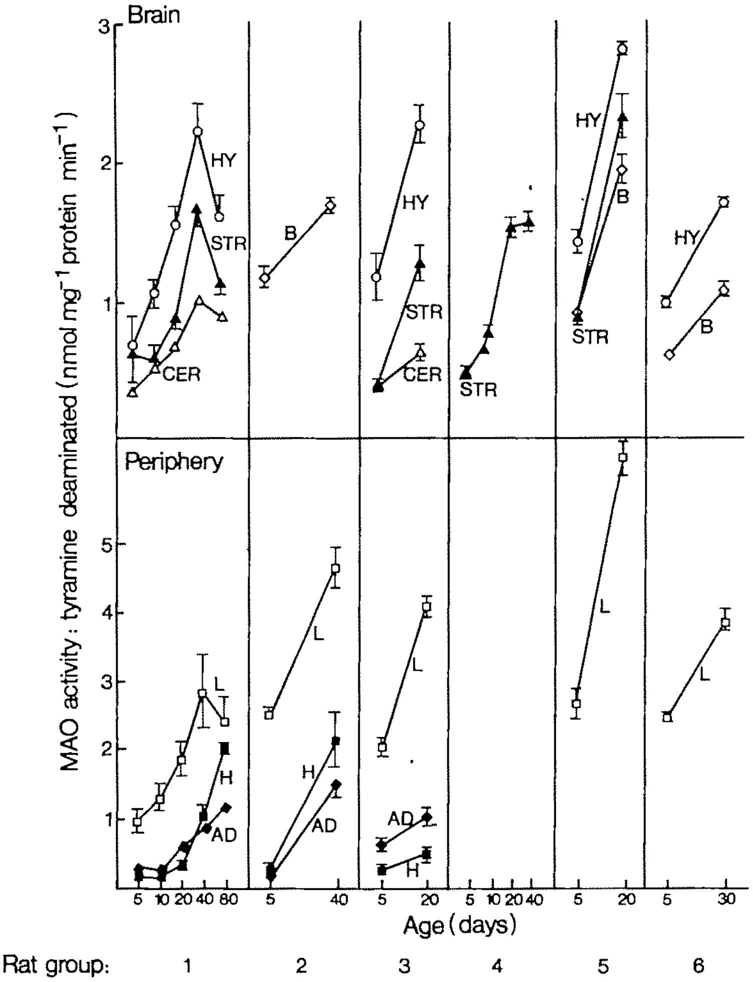

Figure 3 Postnatal development of monoamine oxidase (MAO) activity towards tyramine (nmol deaminated mg^{-1} protein min^{-1}) in litter-mate male rats aged between 5 and 80 days. Groups 1 and 2: Wistar rats bred in Babraham. Groups 3 and 4: Wistar rats bred at Tuck's. Group 5: Porton rats (bred at Babraham); Group 6: Hooded rats (bred at Babraham). (For definition of groups see also Table 1). Hypothalamus (HY, ○); striatum (STR, ▲); cerebellum (CER, △); whole brain homogenate (B, ◇); liver (L, □); heart (H, ■); adrenal glands (AD, ◆). Mean values are given; vertical lines show s.e. mean; $n=4-5$. (The same tissue homogenates of group 1 were also used for the assays with kynuramine in Figure 2).

tryptamine deaminated per mg protein. Highest activities were seen in group 3. In group 1 the values obtained for the cerebellum and septum were similar to those for the striatum and are therefore omitted from the figure. In group 6 the change in enzyme activity with age in the hypothalamus and striatum went in opposite directions. In the brain homogenates of groups 2 and 5 no significant difference in tryptamine deamination between days 5 and 40 or 5 and 20 could be detected. In contrast to the brain regions tryptamine deamination by the liver increased with age in all groups as seen for the other substrates.

In all groups the quantity of tryptamine deaminated on day 5 was smaller than that of tyramine and the increase with age was less steep. Very little tryptamine was deaminated by the hearts and adrenal glands of the 5–20 day old rats. Deamination increased by day 40 and 80 but the rise was less steep than that for tyramine.

(e) *5-Hydroxytryptamine.* The ability of MAO to deaminate 5-HT at different ages varied between the groups of rats (Figure 6). In group 1 there was a steep fall between days 5 and 40 in all the tissues tested, the

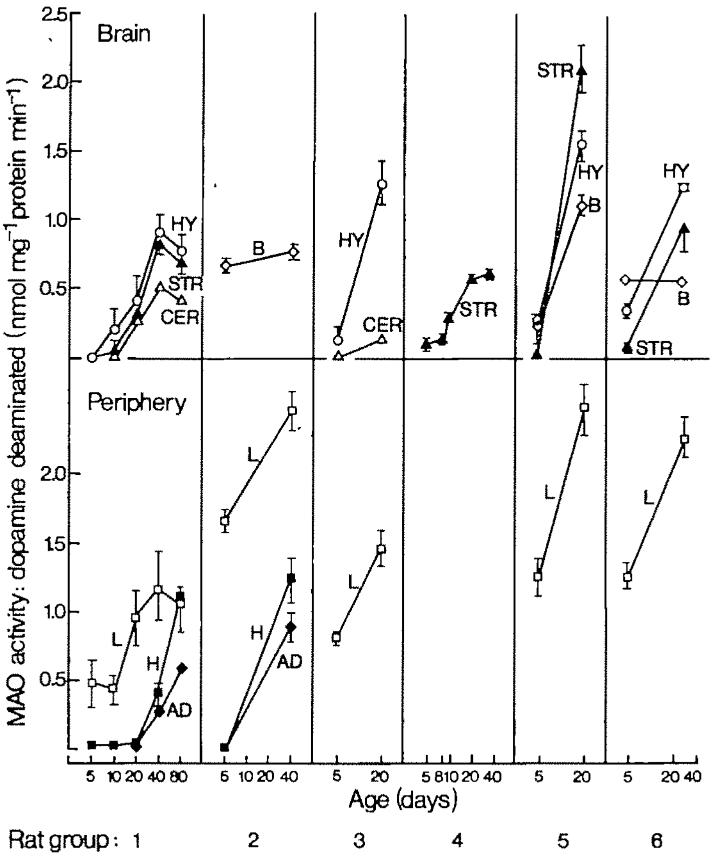

Figure 4 Postnatal development of monoamine oxidase (MAO) activity towards dopamine (nmol deaminated mg^{-1} protein min^{-1}). The same tissue homogenates were tested as in Figure 3. Symbols as in Figure 3.

other groups showed a rise with the exception of the whole brain homogenates of groups 2 and 6 in which no significant difference between day 5 and 40 occurred. The smallest quantities of 5-HT were deaminated by the hearts and adrenal glands of groups 2 and 3. More 5-HT than tyramine was deaminated by the hypothalami of the 5 day old rats in groups 1 and 3, less in groups 5 and 6.

II. Postnatal development of multiple forms of monoamine oxidase

1. *Differences in the development of a tyramine and a dopamine deaminating enzyme system in the rat brain.* Figure 7 illustrates the increase with age in the activity of MAO towards three different substrates in the hypothalami of the rats of group 1. The activities are expressed in percent of the highest activity observed in the 40 day old rats. MAO activity towards kynuramine and tyramine was doubled between days 10 and 40 whereas activity towards dopamine increased four-fold during the same period. This observation suggested the possibility that in the hypothalamus the enzyme system deaminating dopamine might develop with age independently from the tyramine deaminating system. Similar observations were made in other groups of rats. In the rats of group 3 the dopamine MAO activity in the hypothalamus on day 20 was about eight times higher than on day 5 (see Figure 4) whereas tyramine MAO activity only doubled during the same time (see Figure 3). In group 5 (Porton strain) dopamine deamination by the hypothalamic and striatal tissues on day 21 was seven to eight times that on day 5 but the increase in tyramine deamination was only two-fold.

In the hearts and adrenal glands a differential development of dopamine and tyramine deamination also became apparent. Deamination of tyramine by both tissues was slow but clearly demonstrable in 5, 10 and 20 day old rats. In the same homogenates no dopamine deamination could be detected. Between

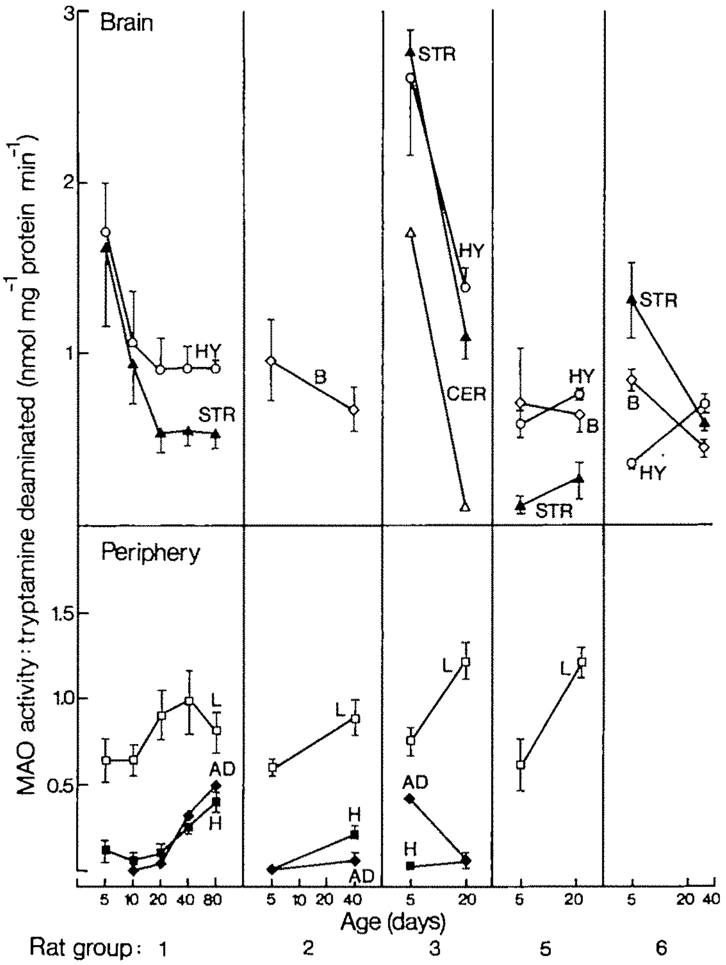

Figure 5 Postnatal development of monoamine oxidase (MAO) activity towards tryptamine (nmol deaminated mg^{-1} protein min^{-1}). The same tissue homogenates were tested as in Figures 3 and 4. Symbols as in Figure 3.

days 20 and 80 tyramine deamination in the heart increased six-fold, dopamine deamination however increased more than twenty-fold. In the adrenal gland there was a two-fold rise in tyramine deamination between days 20 and 80 in contrast to a more than twenty-fold rise in dopamine deamination (see Figures 3 and 4).

It was surprising to see that in group 2 and in group 6 (Hooded rats) the dopamine deamination in extracts of whole brain did not increase with age whereas it did so in the hypothalamic homogenates (Figure 4). In the same rats the rise in tyramine inactivation by the whole brain homogenate was similar to the rise in tyramine inactivation in the hypothalamus (Figure 3).

In the livers of all groups studied tyramine and dopamine deamination developed at a similar rate. Thus in groups 1, 3 and 5 both tyramine and dopamine deamination increased two-fold between days 5 and 20.

Assuming that tyramine and dopamine were deaminated by the same enzyme system one would expect the ratios of MAO activities expressed as nmol amine deaminated mg^{-1} protein min^{-1} towards these two substrates to remain constant during development in a given tissue. From Figure 8 it can be seen that this is in fact the case for the liver. In all groups of rats the ratio of tyramine to dopamine deaminated was about 2 at all ages tested. The whole brain homogenates of 5 day old rats also deaminated only about twice as much tyramine as dopamine, as did the livers. In contrast, in the hypothalamus and striatum of 5 day old rats this ratio was much larger (between 4 and >10) than in the same tissues of 20 to 80 day old rats when it was similar to that in the liver.

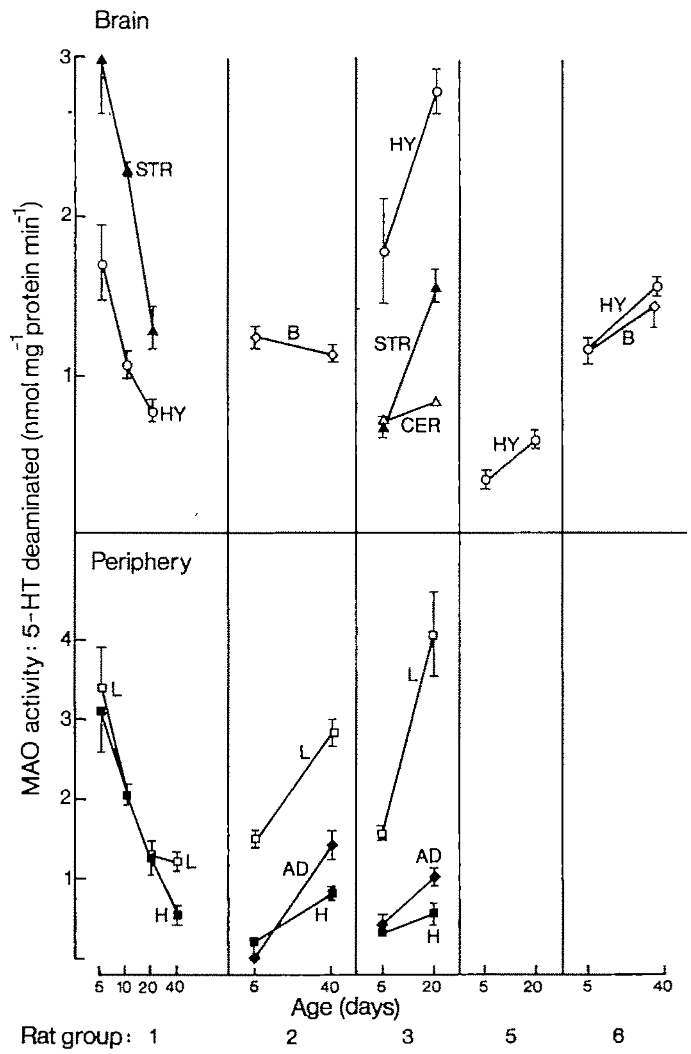

Figure 6 Postnatal development of monoamine oxidase (MAO) activity towards 5-hydroxytryptamine (5-HT) (nmol deaminated mg^{-1} protein min^{-1}). The same tissue homogenates were tested as in Figures 3, 4 and 5. Symbols as in Figure 3.

In the homogenates of the hearts and adrenal glands no dopamine metabolism could be detected in the 5 day old rats and on day 20 only on rare occasions. At these ages the ratios of tyramine deaminated to dopamine deaminated were well over 20.

2. *Independent development of a tryptamine deaminating enzyme system in the rat striatum and hypothalamus.* A comparison between the results in Figure 3 and Figure 5 leaves little doubt that the enzyme deaminating tryptamine develops in the young rat differently from the enzyme deaminating tyramine. Figure 9 shows the ratios between tyramine deamination to tryptamine deamination in the hypothalami and livers of 3 groups of rats. Because of the rise in tyramine and the fall in tryptamine deamination with age these ratios increased with age. In group 1 the ratios obtained for the septum, the cerebellum and the striatum (not shown in the figure) were the same as those for the hypothalamus. The ratios also increased in the livers because the rise with age of tyramine deamination was faster than that of tryptamine deamination. Similar changes in the tyramine : tryptamine ratios were seen in the hearts and adrenal glands.

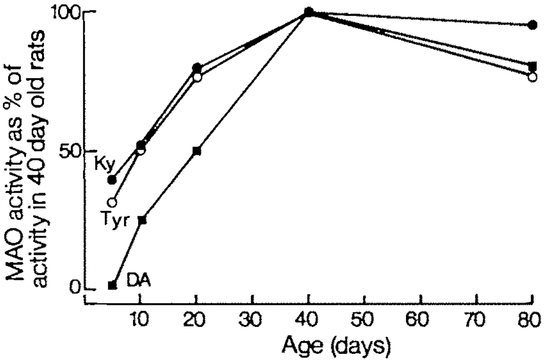

Figure 7 Monoamine oxidase (MAO) activity towards kynuramine (Ky), tyramine (Tyr) and dopamine (DA) in the hypothalamus of the rats of group 1 (see Figures 2, 3, 4 and Table 1) at different ages expressed as percent of the highest activity observed in the 40 day old rats. Kynuramine (Ky, ●); dopamine (DA, ■); tyramine (Tyr, ○).

III. Subcellular distribution of monoamine oxidase activity in the hypothalamus of 5 and 40 day old Porton rats

MAO activity was estimated (see methods section) in subcellular fractions of hypothalamic tissue of male rats belonging to the Porton strain which were killed when 5 or 40 days old. The results are shown in Figure 10. Per mg protein, the largest quantities of all the amines tested were deaminated by a mitochondrial fraction which sedimented at about 1.5 M on the sucrose gradient (P_2). This fraction showed in the 40 day old rats, per mg protein, about 1.7 times more MAO activity towards tyramine, 1.6 times more activity towards dopamine and 1.2 times more activity towards 5-HT than in the 5 day old rats. There was no difference with age in the tryptamine deamination. MAO activity was also found in the P_1 fraction (nuclear) and was higher in the 40 than in the 5 day old rats for all substrates. Activity in the combined supernatant and microsomal fraction was very low and did not increase with age.

IV. Comparison of monoamine oxidase activity in the liver with that in the hypothalamus and heart

Per mg protein the liver deaminated on average 1.5 times more kynuramine and tyramine than the hypothalamus. This was seen in most breeding colonies at all ages studied. For dopamine the figure was about 2 in rats aged 20 days or older. At an earlier age dopamine deamination in the hypothalamus was very low (see Figure 4). The hepatic deamination of the two indolalkylamines was equal to or somewhat lower than in the hypothalamus in rats older than 10 days.

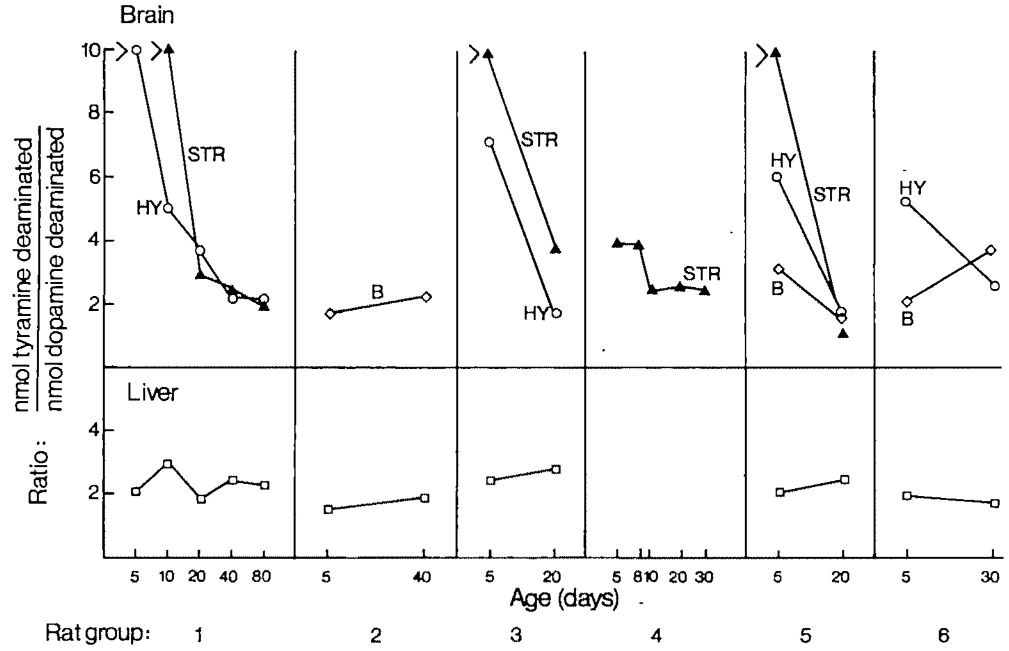

Figure 8 Variations with age in the ratio of monoamine oxidase (MAO) activity towards tyramine to MAO activity towards dopamine in the hypothalamus (○), striatum (▲), whole brain homogenates (◇) and livers (□) of all rats tested (compare values of Figures 3 and 4).

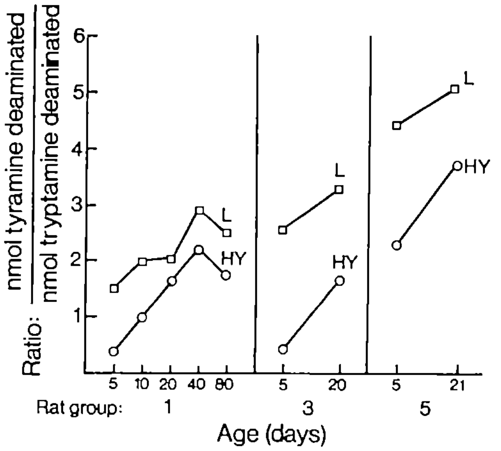

Figure 9 Variations with age in the ratio of monoamine oxidase (MAO) activity towards tyramine to MAO activity towards tryptamine in the hypothalamus (HY,○) and livers (L,□) of the rats of groups 1, 3 and 5.

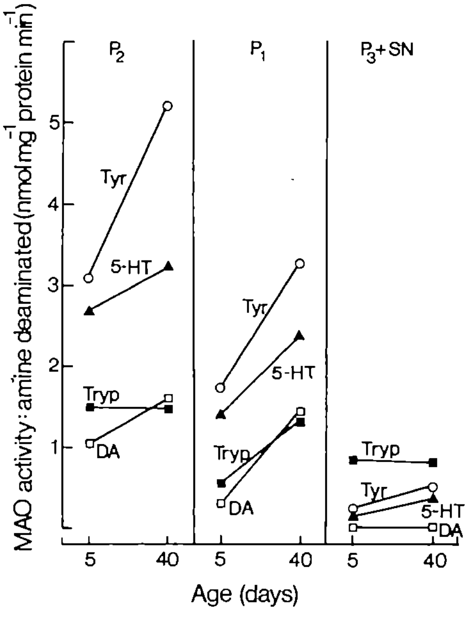

Figure 10 Monoamine oxidase (MAO) activity in subcellular fractions of the pooled hypothalami of either 5 (n=10) or 40 (n=4) day old Porton rats. P_2=purified mitochondrial fraction; P_1='nuclear' fraction; P_3='microsomal' fraction; SN: high speed supernate. MAO activity expressed as nmol substrate deaminated mg^{-1} protein min^{-1}; substrates: tyramine, (Tyr, ○); 5-hydroxytryptamine, (5-HT, ▲); dopamine, (DA, □); tryptamine, (Tryp, ■).

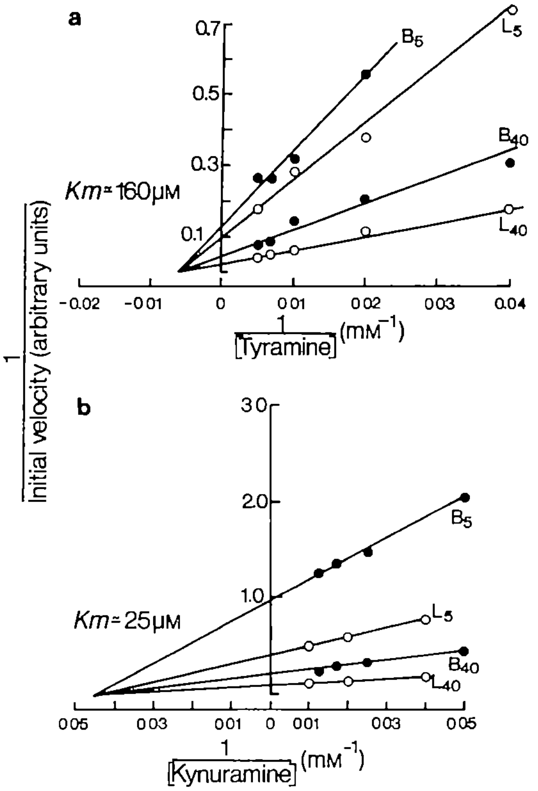

Figure 11 *Km* values for the deamination of (a) tyramine (*Km*≃160 μM) and (b) kynuramine (*Km*≃25 μM) by brain homogenates of 5 day (●, B5) and 40 day (●, B40) old rats and liver homogenates (○, L5; ○, L40) of the same rats.

The time course of the development of MAO towards all substrates studied differed considerably between liver and heart. Up to 40 days MAO activity in the heart was relatively low and in groups 1, 2 and 3 about 10 times more kynuramine, tyramine, dopamine and tryptamine was deaminated per mg protein in the liver than in the heart. At 40 and 80 days this ratio was below 5.

V. Physico chemical properties of monoamine oxidase at different ages

The *Km* values for the deamination of kynuramine and tyramine by liver and whole brain homogenates of 5 and 40 day old rats are shown in Figure 11 (a) & (b). Neither tissue showed a difference in the *Km* values obtained at different ages. For kynuramine the value was 25 μM, and for tyramine 160 μM.

The heat inactivation (48°C) of MAO in various tissues of 5 and 40 day old rats was also tested using

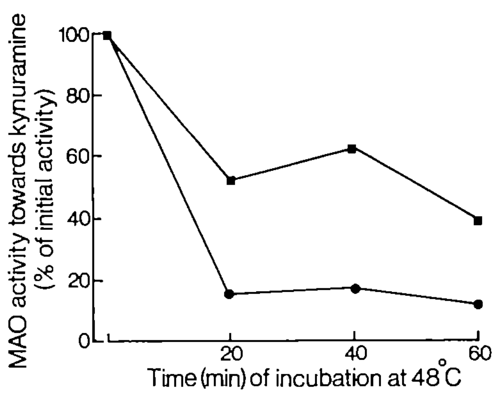

Figure 12 Heat inactivation of monoamine oxidase (MAO) activity towards kynuramine in adrenal homogenates (AD) of 5 (●) and 20 (■) day old litter-mate male rats (Wistar, Babraham bred). Incubation at 48°C in Na-phosphate buffer (0.05 M, pH 7.4).

different substrates. Figure 12 shows that the ability of adrenal homogenates of 5 day old rats to deaminate kynuramine decreased by more than 80% after 20 min whereas in the 40 day old adrenal glands it was decreased by only 50% after the same time. Figure 13 shows heat inactivation curves of MAO in liver and brain homogenates of 5 and 40 day old rats. The loss in enzyme activity was greater in the younger rats. This was especially pronounced when kynuramine was used as substrate. The decreased heat stability of the enzyme in the tissue of young rats may be caused by the absence of stabilizing compounds. In the brain it is known that the lipid content per g tissue increases more than ten-fold between day 12 and 48 (Crawford & Sinclair, 1972; Mead & Dhopeshwarkar, 1972).

VI. Monoamine oxidase activity during the development of the domestic pig

Results obtained on the hippocampus and the adrenal glands are listed in Table 2. In the hippocampus of the foetal pig, the MAO activity towards tyramine and tryptamine was only about one-third of that in the 1 week old piglet and no dopamine (<0.01 nmol mg^{-1} protein min^{-1}) was deaminated. The ability to deaminate dopamine increased gradually with age. The homogenates of the hippocampus of the 6 weeks old piglet deaminated per mg protein about twice as

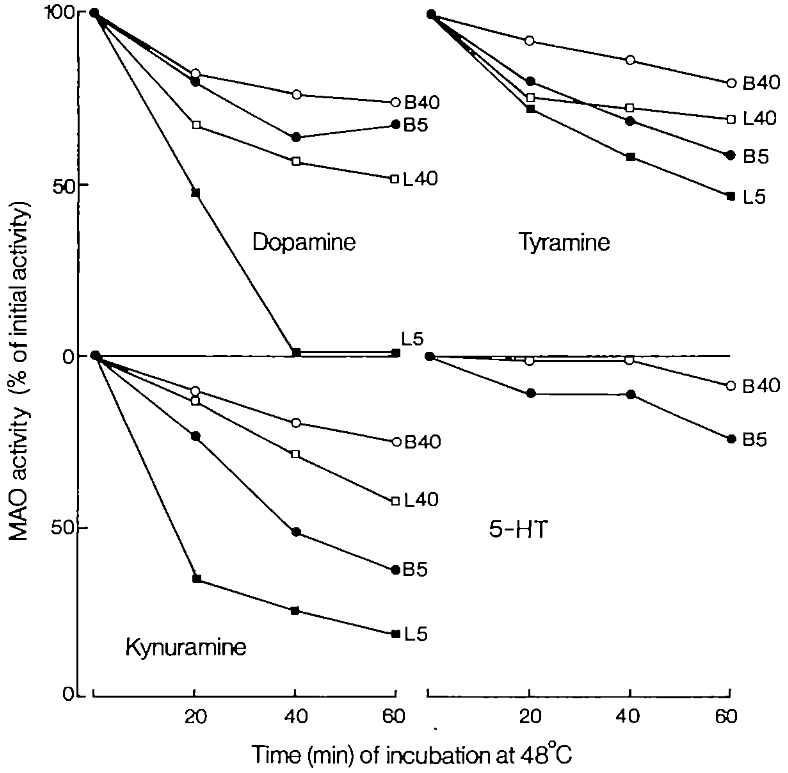

Figure 13 Heat inactivation of monoamine oxidase (MAO) activity in brain (B) and liver (L) homogenates of 5 and 40 day old litter-mate male rats (Wistar, Babraham bred) towards different substrates: (●) 5 day old brain; (O) 40 day old brain; (■) 5 day old liver; (□) 40 day old liver.

much dopamine as that of the 1 week old piglet. There was also a very small rise in MAO activity towards tyramine. Because of large individual variations and the small number of samples available, the changes were statistically not significant. It is of interest that the ratio between the amount of tyramine deaminated to the amount of dopamine deaminated by the hippocampus decreased with age as it did in discrete brain regions of the rat. It was >10 in the foetus, 7.7 in the 1 week old piglet, 7.3 at 2 weeks, 5.1 at 4 weeks and 4.8 in the 6 week old piglet. In two adult pigs (unpublished results) this ratio was found to be 2. No foetal adrenal tissue was available from this litter of pigs. The adrenal MAO activity towards all three substrates was lowest on day 1. Any further increases were not statistically significant.

Discussion

The results of this investigation have elucidated two major points. First, an independent development of at least three enzyme systems deaminating different monoamines has been observed in discrete brain regions, the heart and the adrenal glands, but not in the livers of the rat. Second, there were differences between breeding colonies which were especially pronounced when 5-HT was used as substrate. This finding emphasizes the danger of considering observations made on a single rat colony as representative for the whole species.

The independent development of enzyme systems deaminating one specific monoamine in discrete brain regions but not in the liver could be due to the different chemical environments prevailing in these tissues. Similarly the independent development of MAO activity deaminating tyramine or dopamine or tryptamine does not provide proof that different enzyme proteins are involved in the deamination of these substrates. Developmental changes in some components in the immediate environment of the enzyme protein (allotopic or allosteric effectors) which are required for conformational stability and for the most efficient deamination of a given monoamine could account for the differences observed when various substrates are used to test MAO activity in discrete brain regions.

This point could be carried even further to explain the general phenomenon of increasing enzyme activity with age as seen in most organs towards most substrates. This is not necessarily due to a change in the quantity of enzyme protein present per mg total protein but could result from developmental changes of allotopic or allosteric effectors and co-enzymes. This is exemplified by the postnatal increase in the tissue content of flavin (Kuzuya & Nagatsu, 1969) which is a co-factor of MAO (for review see Youdim, 1976). The activity of the enzyme effecting flavin synthesis is known to increase with age (Rivlin, Fazekas, Huang & Chaudhuri, 1976). It is also possible that an enzyme responsible for the covalent binding of FAD to the MAO apo-enzyme is not fully developed at birth. Phospholipids situated in close proximity to MAO in the outer mitochondrial membrane are also known to be of importance for certain properties of MAO. It was suggested that interaction between the enzyme protein and phospholipids was responsible for the multiple forms of MAO, for substrate-dependent differences in the rate of its heat inactivation and for inhibitor specificity (Tipton, Youdim & Spires, 1972; Houslay & Tipton, 1973; Tipton, Houslay & Garrett, 1973). Like the

Table 2 Developmental changes of monoamine oxidase (MAO) activity in the piglet*

		MAO activity: (nmol amine deaminated mg^{-1} protein min^{-1})		
Age	*Substrate:*	*Dopamine*	*Tyramine*	*Tryptamine*
	(1) Hippocampus			
Foetus (55 day) ($n=4$)		<0.01	0.24 ± 0.09	0.05 ± 0.03
1 week ($n=4$)		0.10 ± 0.04	0.77 ± 0.15	0.14 ± 0.07
2 weeks ($n=4$)		0.12 ± 0.07	0.88 ± 0.09	0.21 ± 0.09
4 weeks ($n=4$)		0.19 ± 0.05	0.97 ± 0.09	0.13 ± 0.05
6 weeks ($n=2$)		0.21 ± 0.04	1.00 ± 0.07	0.10 ± 0.05
	(2) Adrenal gland			
1 day ($n=4$)		0.19 ± 0.05	0.69 ± 0.11	0.07 ± 0.05
1 week ($n=8$)		0.28 ± 0.03	1.34 ± 0.11	0.20 ± 0.08
2 weeks ($n=6$)		0.32 ± 0.03	0.90 ± 0.15	0.13 ± 0.06
4 weeks ($n=8$)		0.22 ± 0.03	0.91 ± 0.09	0.12 ± 0.04
6 weeks ($n=2$)		0.25 ± 0.04	0.68 ± 0.07	0.11 ± 0.06

* Breed: Large White; n = number of samples.

flavins, the brain lipids are low at birth and increase gradually with age (see e.g. Mead & Dhopeshwarkar, 1972; Crawford & Sinclair, 1972).

MAO forms a constituent of the outer mitochondrial membrane (Schnaitman, Erwin & Greenawalt, 1967). Thus studies on the development and life span of the mitochondria in the organs investigated could be of importance for the understanding of the processes involved in the developmental changes in MAO activity as would be a knowledge of the turnover rate of the MAO protein at various ages. The difficulties in assessing the accurate life span of a mitochondrion are numerous. Most of the measurements are based on the turnover rates of mitochondrial constituents which are known to have different half lives thus indicating that synthesis of different proteins can take place independently in the intact organelle (for review see Munn, 1974). Gregson & Williams (1969) found that in the brain the number of mitochondria per g wet weight does not increase with age, however the activity (per g wet weight) of the two mitochondrial enzymes succinate dehydrogenase and cytochrome oxidase increased four and six-fold respectively. From this work we may infer that after birth the MAO activity (towards tyramine, dopamine and kynuramine) increases per mitochondrion. The interpretation of findings on brain MAO is further complicated by the simultaneous presence of neuronal and glial tissue in the homogenates. Furthermore, Maitre, Delini-Stula & Waldmeier (1976) obtained evidence that MAO may be present in the brain in at least two pools, a 'bulk' MAO and a small MAO pool. The latter is characterized by its relative resistance to inhibition, a rapid turnover and preference for dopamine deamination. It is possible that in discrete brain regions the population of mitochondria deaminating different monoamines develops independently. The existence in the adult rat brain of mitochondrial fractions which vary in their ability to deaminate dopamine or 5-HT has been described by Kroon & Veldstra (1972) and by Youdim (1974).

In contrast to most other organs, the cardiac MAO activity in the rat did not reach maximal values by the end of the 4th month. A study of the development of cardiac MAO activity towards 5-HT has been carried out by Horita (1967) in rats aged 2–16 weeks. The heart of the male rats deaminated per g tissue six times less 5-HT at four weeks than at 16 weeks, when a plateau was reached. The postnatal development of the enzymes succinic dehydrogenase and cytochrome oxidase did not follow the same time course as that of MAO. Callingham & Della Corte (1971) reported that the half life of cardiac MAO increased with age. In rats with a mean body weight of 155 g it was 6.7 days, in rats weighing about 230 g it was 10.2 days and in rats weighing 340 g 17.6 days. These authors found a similar increase with age in the half life of $NADH_2$-cytochrome C reductase. A difference in the postnatal development in the heart of two forms of MAO (type A and B) distinguished by their behaviour towards different inhibitors (Johnston, 1968) has recently been described by Callingham & Lyles (1975).

The observation made on the rats of group 1 that MAO activity mg^{-1} protein min^{-1} towards 5-HT decreases during the first 20 days after birth was in conflict with the results in all other groups studied and with previous reports in the literature (Karki *et al.*, 1962; Bennett & Giarman, 1965; Horita, 1967). We ascribe it to differences between breeding colonies.

The observation that dopamine was hardly metabolized by the homogenates of hypothalami, striata, heart and adrenal glands of 5 and 10 day old rats of all groups studied seems of special physiological interest. The fact that the whole brain homogenates from 5 day old rats metabolized considerable quantities of dopamine in contrast to striata and hypothalami draws attention to the importance of analysing discrete brain regions.

There was also a discrepancy in the findings on the development of dopamine deamination by homogenates of the hypothalamus and by purified hypothalamic mitochondria. MAO activity towards dopamine in the homogenates of hypothalami increased seven-fold between days 5 and 21. In contrast in the purified hypothalamic mitochondria of rats of the same colony there was only a 1.5-fold increase between days 5 and 40. Is it possible that in the homogenates of the hypothalami of 5 day old rats an enzyme inhibitor is present which is being removed during the purification of the mitochondria? A localized enzyme inhibitor in the brain of young rats may also be responsible for the observation that MAO activity per mg protein towards dopamine in the homogenates of whole brains of 5 day old rats was much higher than in the isolated hypothalami or striata and that there was no increase with age. In the whole brain homogenates a localized inhibitor of dopamine deamination might have become so diluted that it was no longer effective.

The development of MAO activity towards dopamine in the striata appears to follow the maturation of the neuronal system employing dopamine as a transmitter substance. In a detailed fluorescence histochemical study, Loizou (1972) observed that during the first week after birth the presumed dopamine containing varicosities were sparse whereas by the end of the third week the innervation was so dense that it was no longer possible to distinguish the individual varicosities.

Is it possible to draw from the experiments on tissue homogenates *in vitro* any conclusions about the *in vivo* function of MAO during development? This question has been investigated for dopamine deamination by measuring the MAO activity towards dopamine and simultaneously the two acidic metabolites of dopamine, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) in the striata of littermate rats aged 5–30 days. When expressed as percent of the values obtained on day 5 the rise with age in the striatal concentrations of HVA and DOPAC and the increase in the deamination of dopamine was similar (Davis *et al.,* 1975). Thus, at least for dopamine deamination it appears that the results of the *in vitro* experiments may reflect the *in vivo* situation. However, this may be a purely fortuitous occurrence as the development of dopamine deamination *in vitro* followed a similar time course with age in other brain regions (e.g. the cerebellum) in which no extensive dopamine containing systems have so far been described. This was also true for the adrenal glands and the heart, but not for the liver.

There are also differences between species in the increase of MAO activity during postnatal development. Thus, Karki *et al.* (1962) found that the ability of the brain of the rat to deaminate 5-HT was three times higher in the adult rat than in the newborn rat, whereas in the guinea-pig this difference between the adult and the newborn animal did not exist. Likewise, Horita (1968) was unable to find a rise with age in cardiac MAO activity towards 5-HT in the guinea-pig, rabbit, cat and mouse. In the adrenal gland and the hippocampus of the domestic pig the rise with age in MAO activity towards dopamine, tyramine and tryptamine was also much less pronounced than in the rat (see Table 2). However, the domestic pig has reached a much higher degree of maturation at birth than the rat and many developmental changes which occur in the rat during the first weeks of life will have taken place *in utero* in the pig. The decrease with age in the ratio of the quantity of dopamine to tyramine deaminated by the pig hippocampus could indicate that, as in the rat, the development of a dopamine deaminating system may take a different time course from a tyramine deaminating system. In certain regions of the pig brain, in which dopamine may play a role in neuronal transmission, the concentration of homovanillic acid increased during the first 6 weeks after birth (Fry, Sharman & Stephens, 1976) as has also been found in the rat (Davis *et al.,* 1975). Stanton, Cornejo, Mersmann, Brown & Mueller (1975) found higher MAO activity towards tyramine in the 'hind Brain' of pigs (crossbred: Hampshire X Yorkshire X Duroc), between birth and 70 days than at 150 days. Tyramine deamination by the adrenal glands of these pigs remained apparently constant during the first 5 months.

In order to explore the possibility that the differential deamination of specific monoamines by MAO is of physiological significance, analysis will have to be conducted on very small brain regions (individual nuclei) and simultaneous measurements made of the enzymes responsible for the synthesis of a given monoamine, the tissue concentration of that amine and those of its catabolic products and the activity of the enzymes responsible for the fromation of these catabolites.

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COMPARATIVE EFFECTS OF PROPRANOLOL AND PRACTOLOL IN THE EARLY STAGES OF EXPERIMENTAL CANINE MYOCARDIAL INFARCTION

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1 The effects of propranolol and practolol, at equivalent myocardial β-adrenoceptor blocking doses, (as assessed by the degree of shift of isoprenaline dose-response curves) were investigated in anaesthetized greyhounds before and after acute coronary artery ligation.

2 When administered intravenously to the intact closed-chest dog, propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) caused similar decreases in heart rate, left ventricular dP/dt *max*, myocardial blood flow and cardiac output. Only propranolol increased peripheral vascular resistance.

3 When administered 2–3 h after acute coronary artery ligation, propranolol (0.1 mg/kg) significantly decreased blood flow in both normally perfused and ischaemic regions of the heart. There was also electrocardiographic evidence of further deterioration after propranolol; two out of seven animals died following this treatment.

4 Practolol (0.5 mg/kg) when administered after coronary artery ligation also decreased normal myocardial blood flow but flow in the ischaemic area remained unchanged. Evidence was obtained from electrocardiographic, myocardial temperature, myocardial O_2 consumption and lactate measurements that the administration of practolol, in contrast to propranolol, benefited the ischaemic myocardium.

5 Analysis of the results suggests that this beneficial action of practolol may be related to at least two mechanisms. Firstly the ability of practolol to increase the period during diastole when perfusion of the subendocardium is possible, without decreasing the transventricular pressure during this period. Secondly that practolol does not unmask α-adrenoceptor vasoconstriction in the ischaemic region.

Introduction

The suggestion that β-adrenoceptor blockade with propranolol offers some protection against the development of a subsequent experimental myocardial infarct was first made by Grayson, Irvine, Parratt & Cunningham (1968). Their findings in dogs were confirmed and extended by Maroko, Kjekshus, Sobel, Watanabe, Covell, Ross & Braunwald (1971) and by Serrano, Chavez-Lara, Bisteni & Sodi-Pallares (1971) who assessed the degree and extent of ischaemic injury from epicardial electrocardiograms. Similar results have been obtained with the cardio-selective β-adrenoceptor antagonist, practolol (Libby, Maroko, Covell, Malloch, Ross & Braunwald, 1973) and it has also been demonstrated (Pelides, Reid, Thomas & Shillingford, 1972) that this drug is capable of reducing 'infarct size' in patients shortly after the onset of acute myocardial infarction. Although these beneficial effects of β-adrenoceptor antagonists are thought to be related to a more favourable balance between myocardial O_2 supply and demand in the ischaemia area, there have been no studies published in which these parameters have been measured. There also exists some controversy as to whether 'cardio-selective' β-blockers possess any real advantages over propranolol in the treatment of ischaemic heart disease. In this study we have compared the effects of equivalent myocardial β-adrenoceptor blocking doses of propranolol and practolol in an experimental model which allows the simultaneous measurement of blood flow in both normal and acutely ischaemic regions of the canine heart and which, through selective coronary venous blood sampling, allows comparisons to be made of O_2 handling and metabolism in these areas (Marshall, Parratt & Ledingham, 1974). Some of the preliminary results obtained with practolol have been published (Marshall & Parratt, 1974a).

Methods

Twenty-six greyhounds of either sex and weighing between 22 and 31 kg were used in this study. Anaesthesia was induced with sodium thiopentone (20 mg/kg, injected intravenously) and was maintained, after endotracheal intubation, with 0.5–0.8% trichlorethylene vaporized from a Tritec vaporizer (Cyprane Ltd.). The animals were maintained on intermittent positive pressure ventilation with O_2 using a Palmer pump; the stroke volume was adjusted to give an arterial $P\text{CO}_2$ of 34–41 mmHg; 1 mmHg = 1.333 mbar. Temperature was measured in the mid-oesophagus using a direct recording thermocouple (Ellab, Copenhagen). Under fluoroscopic control, polyethylene catheters were inserted to lie in the descending aorta (via a femoral artery), the right atrium (via a femoral vein) and in the coronary sinus (via the left jugular vein). These catheters were used for the sampling of blood and for pressure measurements using Elema-Schönander capacitance transducers. Left ventricular pressure was measured with a catheter-tip transducer (Millar Instruments, Houston, Texas) inserted by way of the left carotid artery. The frequency response of this catheter system is flat to at least 200 Hz. Left ventricular pressure was also recorded at high gain for the measurement of end-diastolic pressure (LVEDP) and the rate of change of the full left ventricular pressure curve with time (dP/dt) was continuously monitored by means of an Elema-Schönander differentiating circuit.

All capacitance transducers were calibrated before the experiment with a mercury manometer and were zeroed at the level of the animal's right atrium. Mean pressures were obtained by electronic integration. The catheters were kept filled with normal saline to which was added sodium bicarbonate (100 mEq/l), to prevent any gradual onset of acidosis, and heparin (2500 units/l) to prevent clotting. After the position of the intracardiac catheters had been checked fluoroscopically, heparin (1000 units) was injected into the right atrium. Cardiac output was measured by dye-dilution. Indocyanine green (2.5 mg) was injected into the right atrium and blood was withdrawn at a constant rate from the descending aorta through a densitometer (Waters Co., Rochester, Minnesota). The full details of this method have been outlined elsewhere (Douglas, MacDonald, Milligan, Mellon & Ledingham, 1975).

In 11 of the animals (5 given propranolol, 6 given practolol), myocardial blood flow was measured without thoracotomy by the ^{133}xenon clearance technique (Ross, Ueda, Lichtlen & Rees, 1964; Ledingham, McBride, Parratt & Vance, 1970). A Sones 7F catheter was introduced into the right common carotid artery in the neck and, under fluoroscopic control, manipulated until the tip of the catheter lay a distance of 5–10 mm into either the circumflex or anterior descending branch of the left coronary artery. Injections of ^{133}xenon (40–100 µCi, dissolved in 0.9% w/v NaCl solution (saline)) were flushed into this catheter with 3 ml heparinized saline warmed to 37°C. The clearance of xenon from the myocardium (which is a function of capillary blood flow) was measured by means of a narrowly collimated Ecko scintillation counter clamped over the praecordium, an Ecko ratemeter (operating with a 3 s time constant) and a Servoscribe pen recorder. Myocardial blood flow per 100 g tissue was calculated from the clearance curve as previously described (Ledingham *et al.*, 1970).

The remaining 15 dogs were subjected to thoracotomy, the heart was exposed and the anterior descending branch of the left coronary artery (LAD) was prepared for ligation at a position distal to the main septal branch. Blood draining from the potentially ischaemic portion of the heart was sampled by means of a small catheter inserted into one of the anterior coronary veins which lie parallel to the artery. Blood obtained from the aorta, the right atrium, the coronary sinus and the local coronary vein was analysed for $P\text{O}_2$, $P\text{CO}_2$, pH and O_2 content as previously described (Marshall *et al.*, 1974). Samples were also analysed for lactate by a standard spectrophotometric technique (Hohorst, 1962). Blood flow in the left circumflex coronary artery was measured with a Nycotron 372 electromagnetic flowmeter and an implantable 2.0 or 2.5 mm flow probe.

After a 1–2 h stabilization period, the LAD was ligated in one stage. A Portex catheter (o.d. 1.34 mm) was inserted into the ligated vessel distal to the ligature. The pressure in this ligated vessel (peripheral coronary pressure; PCP) was measured by means of an Elema-Schönander capacitance transducer. This coronary artery catheter was also used for the measurement of retrograde coronary flow (backflow from the open catheter) and for the injection of small amounts (usually 20 µCi) of radioactive ^{133}xenon. Clearance of radioactivity from the clearly demarcated ischaemic region was measured with a collimator having a narrow angle of acceptance. This experimental model thus allows the simultaneous measurement of blood flow in both normal (left circumflex flow) and acutely ischaemic (xenon clearance) regions of the canine heart and also allows comparison to be made of coronary sinus blood, draining essentially normal myocardium, with local coronary vein blood, which drains predominantly from the damaged ischaemic zone.

Local myocardial muscle temperatures were measured with small direct-recording copper-constantan thermocouples (Ellab, Copenhagen), anchored at known depths in the ventricular wall, and were compared with the temperature of the aortic blood. During temperature recording care was taken to avoid procedures, such as blood sampling, which might influence local muscle temperature. The depth of insertion of each thermocouple was carefully measured at the end of each experiment.

Systemic arterial pressure, right atrial pressure, left ventricular pressure and d*P*/d*t*, peripheral coronary pressure, left circumflex coronary blood flow and the electrocardiogram (standard limb lead II) were recorded on an Elema-Schönander ink-jet writing recorder (Mingograph 81). The heart rate was measured from the electrocardiogram. O_2 consumption, availability and extraction, coronary and peripheral vascular resistances, and external cardiac work were calculated as previously described (Marshall & Parratt, 1973a). Drugs used were (±)-propranolol hydrochloride and (±)-practolol (ICI), dipyridamole (Boehringer) and (±)-isoprenaline hydrochloride (Pharmax). All doses refer to the free base and the drugs were injected into a catheter in a brachial vein. All values quoted in the text are means ± standard error. Statistical analysis of the results was performed using the Student's *t* test for paired data.

Results

The effects of propranolol and practolol in the closed-chest anaesthetized dog

It was of some importance to investigate the effects of propranolol and practolol at equivalent β-adrenoceptor blocking doses. Preliminary experiments showed that propranolol, 0.1 mg/kg intravenously, caused 11, 16 and 19-fold parallel shifts in the mean dose-response curves for the positive chronotropic, positive inotropic and the vasodepressor responses to intravenous isoprenaline. An equivalent degree of myocardial adrenoceptor blockade was produced by practolol in a dose of 0.5 mg/kg intravenously. When administered in this dose, practolol caused a 17-fold shift in the myocardial dose-response curves to isoprenaline (β_1 responses) but had much less effect (four-fold shift) on isoprenaline-induced vasodepression (a β_2 response).

The haemodynamic effects of propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) in anaesthetized greyhounds without thoracotomy are shown in Table 1. Both drugs caused significant and comparable decreases in heart rate, left ventricular d*P*/d*t* *max*, cardiac output, external cardiac work, myocardial blood flow and myocardial O_2 consumption. Neither drug significantly affected blood pressure or stroke volume. Only propranolol significantly increased LVEDP and coronary and peripheral vascular resistance. The ratio between myocardial O_2 consumption and availability remained unchanged after both propranolol (0.55 ± 0.07 to 0.58 ± 0.07) and practolol (0.60 ± 0.03 to 0.58 ± 0.05).

The effects of coronary ligation

The effects of ligation of the anterior descending branch of the left coronary artery in these experiments were similar to those previously described (Marshall & Parratt, 1973a, Marshall *et al.*, 1974) and included marked decreases in cardiac output and external cardiac work. These changes are secondary to depressed myocardial contractility (manifested by

Table 1 The haemodynamic effects of propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) in the closed-chest greyhound

	Propranolol $n=5$		*Practolol* $n=6$	
	Pre-drug	*15 min Post-drug*	*Pre-drug*	*15 min Post-drug*
Systolic blood pressure (mmHg)	145 ± 13	145 ± 17	180 ± 20	159 ± 23
Diastolic blood pressure (mmHg)	105 ± 8	106 ± 12	126 ± 12	112 ± 19
Heart rate (beats/min)	203 ± 25	149 ± 14*	224 ± 15	162 ± 5*
Cardiac output (litres/min)	4.7 ± 0.5	3.0 ± 0.4*	6.2 ± 1.2	4.5 ± 1.0*
LV d*P*/dt *max* (mmHg/s)	3640 ± 247	2740 ± 317*	4400 ± 601	2500 ± 184*
LVEDP (mmHg)	7.2 ± 0.4	11.0 ± 1.5*	8.1 ± 0.7	9.3 ± 0.4
External cardiac work (kg. m/min)	8.7 ± 0.6	4.9 ± 0.6*	12.5 ± 3.2	8.4 ± 2.7*
Myocardial blood flow (ml 100 g^{-1} min^{-1})	96 ± 23	76 ± 20*	134 ± 20	102 ± 21*
Myocardial O_2 consumption (ml 100 g^{-1} min^{-1})	18.0 ± 2.5	12.3 ± 1.4*	24.0 ± 3.9	15.2 ± 2.1*
Coronary vascular resistance (units)	1.4 ± 0.2	1.8 ± 0.2*	1.1 ± 0.1	1.3 ± 0.2
Peripheral vascular resistance (units)	37 ± 6	59 ± 14*	25 ± 4	28 ± 3

Values are mean ± s.e.
*$P < 0.05$

transient decreases in left ventricular d*P*/d*t* *max* with more sustained increases in LVEDP). Blood flow through the ischaemic region of the myocardium, as measured by radioactive xenon clearance, was about 25% of that from the same region before ligation of the artery.

Haemodynamic effects of propranolol and practolol after acute coronary artery occlusion.

The haemodynamic effects of both propranolol and practolol, when administered 2–3 h after occlusion, were essentially similar to those seen when the drugs were administered before ligation (Table 2). Both drugs decreased heart rate, cardiac output, left ventricular d*P*/d*t* *max* and external cardiac work; LVEDP and coronary and peripheral vascular resistance were increased. Only after occlusion did propranolol significantly decrease systemic blood pressure and stroke volume: Blood flow in the normal myocardium was significantly decreased by propranolol (by a mean of 46%) and there was also a reduction in O_2 consumption (Table 3). Although practolol also markedly decreased cardiac output and external cardiac work, the decreases in blood flow (32%) and O_2 consumption in the normal myocardial regions were less than those seen with propranolol.

Effects of propranolol and practolol in the ischaemic myocardium

Blood flow in the ischaemic region of the myocardium was greatly reduced by propranolol (mean of 54%) as was O_2 consumption (Table 3) and O_2 availability.

Table 2 The effects of propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) on haemodynamics when administered 1–3 h after acute coronary artery ligation

	Propranolol *n* = 7		*Practolol* *n* = 8	
	Pre-drug	*15 min Post-drug*	*Pre-drug*	*15 min Post-drug*
Mean blood pressure (mmHg)	126 ± 7	102 ± 9*	120 ± 7	109 ± 8
Systolic PCP (mmHg)	58 ± 9	48 ± 8	55 ± 7	47 ± 6
Diastolic PCP (mmHg)	20 ± 3	20 ± 3	18 ± 5	17 ± 3
Heart rate (beats/min)	180 ± 14	144 ± 5*	198 ± 13	151 ± 7†
Cardiac output (litres/min)	1.8 ± 0.3	0.8 ± 0.1†	2.0 ± 0.3	1.2 ± 0.2†
Stroke volume (ml/beat)	9.5 ± 1.5	5.7 ± 0.5*	10.1 ± 1.2	7.9 ± 1.2
External cardiac work (kgm/min)	3.3 ± 0.5	1.2 ± 0.7†	3.4 ± 0.6	1.8 ± 0.4†
LV d*P*/d*t* *max* (mmHg/s)	2130 ± 329	1224 ± 143*	2412 ± 324	1288 ± 172†
LVEDP (mmHg)	13 ± 2	21 ± 3†	12 ± 5	17 ± 5
Peripheral vascular resistance (units)	87 ± 13	131 ± 15*	71 ± 16	105 ± 14*

Values are mean ± s.e.
*$P<0.05$; †$P<0.005$.

Table 3 The effects of propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) on blood flow and O_2 consumption in normal and acutely ischaemic regions of the canine myocardium

	Propranolol *n* = 7		*Practolol* *n* = 8	
	Pre-drug	*15 min Post-drug*	*Pre-drug*	*15 min Post-drug*
Normal region of the left ventricular wall				
Blood flow (ml/min)	68 ± 12	37 ± 8*	49 ± 8	33 ± 4*
O_2 consumption (ml/min)	9.7 ± 2.1	6.5 ± 1.8*	8.7 ± 0.6	6.4 ± 0.9*
Coronary vascular resistance (units)	2.0 ± 0.3	3.4 ± 0.5*	2.6 ± 0.3	3.2 ± 0.4
Ischaemic region of the left ventricular wall				
Blood flow (ml 100 g^{-1} min^{-1})	24 ± 7	11 ± 3*	28 ± 6	28 ± 5
O_2 consumption (ml 100 g^{-1} min^{-1})	3.0 ± 1.1	1.6 ± 0.4*	4.9 ± 1.1	4.6 ± 0.8
Peripheral coronary flow (ml/min)	2.4 ± 0.4	1.6 ± 0.2*	2.2 ± 0.5	2.0 ± 0.5
Infarct vascular resistance (units)	1.1 ± 0.4	2.4 ± 0.7*	0.9 ± 0.3	0.7 ± 0.1

Values are mean ± s.e.
*$P<0.05$.

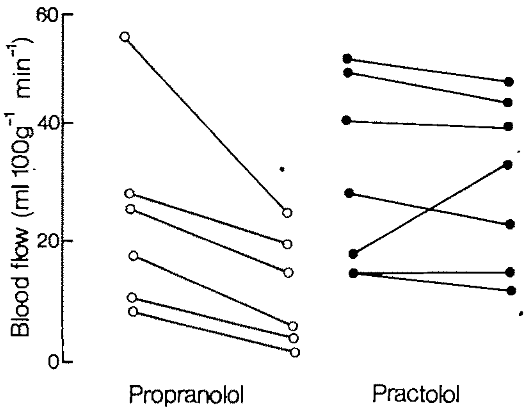


Figure 1 The effects of intravenously administered propranolol (0.1 mg/kg) and practolol (0.5 mg/kg) on blood flow (measured by radioactive xenon clearance) in a region of myocardium made ischaemic by acute coronary ligation, 2 h previously.

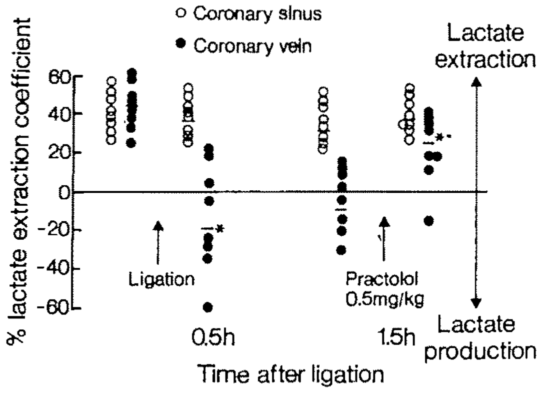


Figure 2 The effects of acute coronary artery ligation and the subsequent administration of practolol on the lactate handling in both normal (O) and acutely ischaemic (●) regions of the canine myocardium. Practolol when administered 2 h after ligation reversed the lactate production to extraction in the ischaemic area. *$P<0.005$.

The further deterioration in blood flow in this critical region was accompanied by a more markedly ischaemic electrocardiographic pattern. The mean ST-segment depression in limb lead II, 2–3 h after ligation in these animals was $570 \pm 80\ \mu V$ and this changed significantly ($P<0.05$) to $890 \pm 120\ \mu V$, 15 min after the administration of propranolol.

In two of the dogs propranolol caused a particularly pronounced bradycardia. This was gradual in onset and progressed to asystole 16 and 23 min respectively after drug administration. As has been previously shown (Marshall *et al.*, 1974), ligation of the LAD results in markedly decreased lactate extraction (or even lactate production) by the region served by that artery with little change in lactate handling by the normal region (Table 4). Propranolol caused no marked changes in lactate handling in either region of the myocardium. (Table 4).

In contrast to propranolol, practolol did not decrease nutritive blood flow in the acutely ischaemic area of myocardium (Figure 1) and O_2 consumption (Table 3) and availability were also unaffected. The back pressure in the ligated artery (PCP) was also unchanged after practolol. There was evidence that practolol improved the situation in the ischaemic myocardium. Thus the ST-segment depression in the electrocardiogram was significantly reduced (from $606 \pm 135\ \mu V$ just before drug administration, to $300 \pm 83\ \mu V$, 15 min after practolol, $P>0.02$). More direct evidence of the beneficial effects of practolol was obtained by analysis of blood lactate and lactate extraction coefficients in both the normal and ischaemic myocardium are shown in Figure 2. Coronary artery ligation caused lactate production by the ischaemic area of myocardium but not by the normally perfused areas draining into the coronary sinus. This shift from aerobic to anaerobic metabolism in the ischaemic myocardium was still evident just before administration of practolol and was reversed by practolol in all but one dog. This means that at least some cells in the ischaemic region were apparently capable of resuming extraction of lactate and this continued for at least 2 h after practolol administration.

Table 4 The effects of ligation, and of the subsequent administration of propranolol, on lactate extraction (%) by both normal (sinus sampling) and acutely ischaemic (venous sampling) regions of the canine myocardium.

Pre-ligation		*30 min Post-ligation*		*2 h Post-ligation*		*15 min Post-propranolol*	
Sinus	*Vein*	*Sinus*	*Vein*	*Sinus*	*Vein*	*Sinus*	*Vein*
41.4	31.9	52.2	−55.0	37.5	−12.9	37.9	11.2
40.6	35.8	30.0	7.2	37.2	13.6	22.0	6.6
32.8	30.5	33.4	6.9	43.0	25.8	31.2	22.0
38.2	35.1	33.2	11.7	35.8	10.1	43.0	6.7

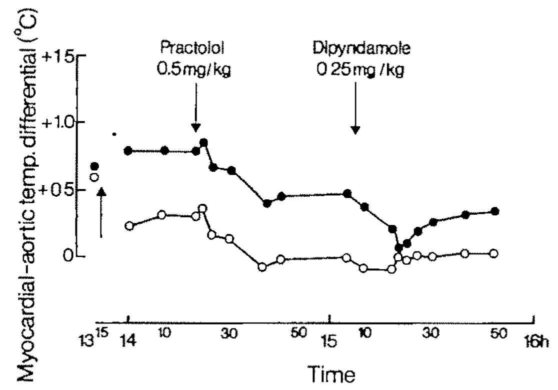


Figure 3 The effects of practolol and the coronary vasodilator drug, dipyridamole, on local myocardial muscle temperature in both normal (●) and ischaemic (O) regions of the canine left ventricle. The arrow indicates time of ligation.

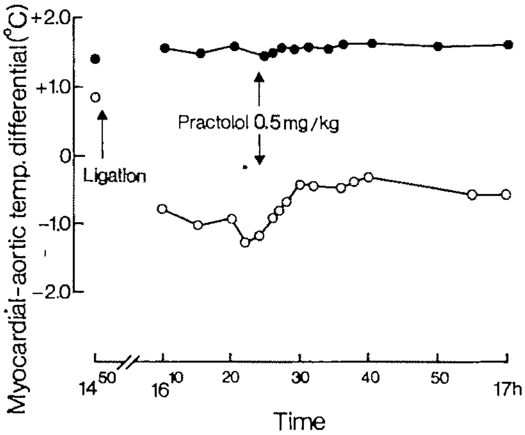


Figure 4 The effects of practolol on both normal (●) and ischaemic (O) myocardial muscle temperature in the deep subendocardial layers of the canine left ventricle.

Effects of propranolol and practolol on local myocardial temperature; comparison with dipyridamole

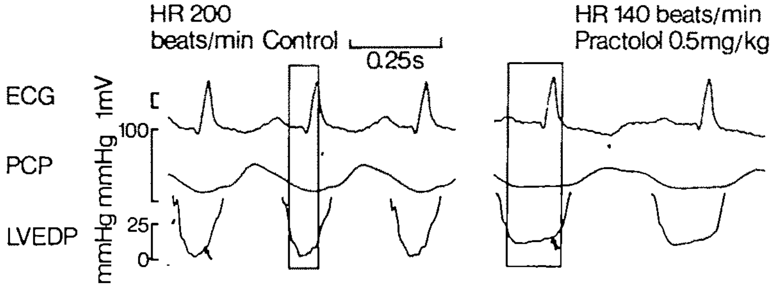
The effects of coronary artery ligation, and of the subsequent administration of practolol, on myocardial temperature are shown in Figure 3. In this particular experiment ligation of the LAD produced a marked fall in temperature in the region (thermocouple depth 6 mm) served by that artery; muscle temperature in a region supplied by the left circumflex branch was slightly increased. Practolol, when administered 1 h after ligation, caused marked decreases in muscle temperature in both the normal and ischaemic zones of myocardium. Similar changes in temperature were obtained in other experiments with both practolol and propranolol. Although these reductions in temperature in the normal myocardium were associated with decreased blood flow and O_2 consumption, only propranolol decreased these parameters in the ischaemic zone (Table 3). The fall in ischaemic muscle temperature produced by practolol cannot therefore be explained by a decrease in O_2 consumption. For comparative purposes, the selective coronary dilator drug dipyridamole was administered. This drug has been shown to increase flow to the normal myocardium considerably and to leave flow in the ischaemic zone unchanged (Marshall & Parratt, 1973b). Dipyridamole also decreased normal muscle temperature, an effect presumably due to increased blood flow. However, in contrast to practolol, dipyridamole did not affect muscle temperature in the ischaemic zone.

Although decreased temperature in both regions of the heart was the usual response to practolol, a different effect was obtained when the thermocouples were placed in the subendocardial region of the ventricle (depth 11–12 mm). The results from one of these experiments are shown in Figure 4. In this particular animal, muscle temperature in the ischaemic region fell markedly on ligation and was 1°C cooler than the aortic blood immediately before the administration of practolol. Muscle temperature in the essentially normal myocardium was relatively unchanged. Practolol did not affect muscle temperature in the normal myocardium but markedly elevated that in the ischaemic region. This increase in muscle temperature could be due to a local increase in subendocardial blood flow, since blood would be a warming factor in this situation.

Discussion

In these experiments, practolol was about 4–5 times less active than propranolol as an antagonist of the positive inotropic and chronotropic actions of intravenous isoprenaline. This agrees well with the results of other workers who, using dogs, have reported ratios of 3–4 (Dunlop & Shanks, 1968), of 2.6 (Boissier, Advenier, Giudicelli & Viars, 1971), and of 4 (Vaughan Williams, Bagwell & Singh, 1973). Our results also confirm previous findings in this species that practolol (in a dose of 0.5 mg/kg) blocks the β_1-adrenoceptor mediated responses to isoprenaline much more effectively than those responses mediated through β_2-adrenoceptors (Dunlop & Shanks, 1968; Ross & Jorgensen, 1970).

The haemodynamic effects of equivalent myocardial β-blocking doses of propranolol and



End-diastolic transventricular pressure (mmHg)	5	7	6	6	6
Effective perfusion period (% cardiac cycle)	26	25	25	32	31
Effective perfusion period/min (s)	32	30	30	41	40

Figure 5 The calculation of end-diastolic transventricular pressure and effective perfusion period in one dog before and 15 min after the intravenous administration of practolol. Although transventricular pressure remains unchanged, the period of effective perfusion is increased by practolol. PCP = peripheral coronary pressure; LVEDP = left ventricular end-diastolic pressure.

practolol in the closed-chest normal dog were very similar except that only propranolol increased peripheral vascular resistance, an effect presumably due to blockade of the β_2-adrenoceptors in peripheral vascular smooth muscle. Certainly the decreases in heart rate, left ventricular d*P*/d*t max*, cardiac output, external cardiac work, myocardial blood flow and O_2 consumption that resulted from the administration of the two drugs were similar in magnitude. This is in contrast to the results obtained when the drugs were administered after acute coronary artery ligation. Although both drugs caused similar decreases in heart rate, left ventricular d*P*/d*t max*, cardiac output and external cardiac work, propranolol tended to reduce blood flow to regions of normal myocardium (mean reduction 46%) rather more than did practolol (32%).

However, a more marked difference between the two drugs became apparent when their effects in the ischaemic region were examined. Propranolol consistently decreased blood flow in this region (by a mean of 54%) whereas practolol had no effect on flow. This consistent finding may be one reason why practolol, but not propranolol, was capable of beneficially modifying other indices of ischaemia, e.g. ST-segment depression and lactate production. There are two possible explanations for this marked difference between the effects of practolol and propranolol in equivalent myocardial β-adrenoceptor blocking doses.

(a) We have previously shown (Ledingham, Marshall & Parratt, 1973; Marshall & Parratt, 1974b) that one of the main factors influencing blood flow in the acutely ischaemic myocardium is the transventricular driving pressure, i.e., the difference during diastole between coronary artery pressure distal to the occlusion and left ventricular pressure. It was therefore of interest to see whether the differing effects of propranolol and practolol on infarct flow were related to changes in this parameter. Propranolol consistently reduced transventricular driving pressure (mean decrease 8 mmHg) whereas practolol had more equivocal effects (the mean change being 3 mmHg). Figure 5 illustrates an example of the calculation of transventricular driving pressure before and after practolol in one dog. Although practolol did not affect the transventricular pressure gradient, the period of effective perfusion (i.e., the period during which diastolic PCP exceeds LVEDP) was increased significantly by the drug. Although heart rate was consistently reduced by practolol, the absolute effective perfusion period was increased by practolol from 24 ± 4 to 42 ± 6 s/min, ($P < 0.05$). Propranolol increased the effective perfusion period to a considerably lesser degree (from 19 ± 2 to 28 ± 3 s/min, $P < 0.05$) and, in contrast to practolol, it actually decreased transventricular driving pressure. Practolol thus significantly increases the period, during diastole, when there exists a positive pressure gradient tending to drive blood to the subendocardial regions of the left ventricular wall. Since this effect would tend to favour blood flow to the subendocardium, it is possible that practolol may cause a redistribution of flow from the epicardial to the endocardial layers of the ischaemic ventricular wall without affecting total blood flow (measured by xenon clearance) in this area. Some experimental evidence for such a flow redistribution was obtained in the present studies from an analysis of temperature records obtained from deeply implanted thermocouples. Practolol markedly elevated temperature in this region (Figure 4), a finding that can be explained on the basis of a local increase in endocardial blood flow. Other experimental evidence for this concept has also been obtained by other workers (Moir & DeBra, 1967; Becker, Fortuin & Pitt, 1971; Fortuin, Kaihara, Becker & Pitt, 1971; Gross & Winbury, 1973). Using either rubidium uptake or radioactive microspheres to measure local regional blood flow, these workers found that β-adrenoceptor antagonists increase the ratio of blood flow between inner and outer layers of the left ventricular wall in both normal and ischaemic regions of the canine myocardium. Such a redistribution of blood flow favouring subendocardial regions would also explain the findings of Winbury, Weiss & Howe (1971) that β-adrenoceptor blockade often produces a selective rise in subendocardial O_2 tension but does not affect, or may even decrease, epicardial O_2 tension.

Possible mechanisms by which β-adrenoceptor antagonists might favour subendocardial perfusion have been recently reviewed by Gross & Winbury (1973) and include decreased heart rate. Of relevance to this problem are the observations that tachycardia *per se* increases 'infarct size' in both anaesthetized (Maroko *et al.*, 1971) and conscious dogs (Shell & Sobel, 1973). In two of the experiments described here in which the dogs were electrically paced, practolol caused a pronounced deterioration of cardiac function. That the bradycardia produced by β-blockers is important to their beneficial effects has also been shown in man (Dwyer, Weiner & Cox, 1967; Balcon, 1971).

(b) The second possible explanation for the difference between the actions of practolol and propanolol on the ischaemic myocardium involves their differential effects on vascular β-adrenoceptors. Most *in vivo* studies indicate either that coronary vascular β-adrenoceptors can be classified as β_2 (Parratt & Wadsworth, 1970; Mark, Abboud, Schmid, Heistad & Mayer, 1972; Adam & Boyles, 1974; Gross & Feigl, 1975) or indicate that they are at least different from myocardial β_1-adrenoceptors (Adam, Boyles & Scholfield, 1970; Ross & Jorgensen, 1970). There is considerable evidence for the release of noradrenaline into the circulation following coronary artery occlusion and, although in the early stages (up to 1 h after occlusion) this amine induces active coronary vasoconstriction in the ischaemic area (for references see Moore & Parratt, 1973), noradrenaline later increases blood flow in the ischaemic region (Marshall & Parratt, 1973a), an effect not solely related to increased perfusion pressure. The administration of propranolol would block vascular β_2-adrenoceptors in the ischaemic region (as it certainly does in the normal coronary circulation; Parratt & Grayson, 1966) and unmask a vasoconstrictor effect of released noradrenaline on vascular α-adrenoceptors present within this region (Parratt, 1967). This would adequately explain the active increase in resistance to flow (from 1.07 ± 0.38 to 2.35 ± 0.48 units; $P < 0.025$) within the developing infarct following propranolol administration. A similar vasoconstriction does not occur after practolol administration (the calculated resistances were 0.86 ± 0.27 units before and 0.68 ± 0.11 units after practolol) presumably because of the relative insensitivity of vascular β-adrenoceptors to this 'cardioselective' blocking agent.

These present studies have shown that at equivalent myocardial β_1-adrenoceptor blocking doses, only practolol benefits an acutely ischaemic area of myocardium; propranolol causes further deterioration which may be fatal. Analysis of the results suggests that the beneficial actions of practolol may be related to at least two mechanisms. Firstly, the ability of practolol to increase the period during diastole when perfusion of the subendocardium is possible without decreasing the transventricular driving pressure during this period. Secondly, the fact that practolol does not unmask α-adrenoceptor vasoconstriction in the ischaemic region. Although practolol also differs from propranolol in that it does not possess direct negative inotropic actions on the heart, it is unlikely that this difference between the drugs is relevant to the results presented here, since 'quinidine-like' properties are only demonstrated with propranolol at considerably higher doses than those used in this study (Harry, Kappagoda, Linden & Snow, 1973). Further evidence excluding this possibility was obtained in this study in the fact that propranolol and practolol decreased left ventricular dP/dt and stroke volume to the same extent.

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IDENTIFICATION OF SEPARATE RECEPTORS FOR ADENOSINE AND ADENOSINE 5′-TRIPHOSPHATE IN CAUSING RELAXATIONS OF THE ISOLATED TAENIA OF THE GUINEA-PIG CAECUM

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1 The mechanisms by which adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine relax the taenia caecum preparation of the guinea-pig have been studied. ATP and ADP produced similar effects which were qualitatively different from those of AMP and adenosine.

2 2-2′Pyridylisatogen tosylate (PIT: 50 μM for 30 min) blocked the effects of ATP and ADP, but exhibited weak activity against AMP and failed to antagonize the effects of adenosine. The action of PIT was unaffected by the inclusion of dipyridamole (2 μM) in the bathing fluid.

3 There was a significant correlation between the sensitivity of individual preparations to ATP or ADP and the blocking potency of PIT.

4 The presence of adenosine in the bathing fluid (2 mM for >30 min) desensitized the taenia to subsequent applications of adenosine. The effects of ATP were increased by this procedure.

5 The results indicate that ATP and adenosine relax the taenia by different mechanisms.

Introduction

Since Burnstock and his colleagues (Burnstock, Campbell, Satchell & Smythe, 1970; Burnstock, 1972) proposed that the chemical mediator of the non-adrenergic inhibitory response to intrinsic nerve stimulation in the gut was adenosine 5′-triphosphate (ATP), there has been renewed interest in the effects of purine derivatives on smooth muscle. A full examination of these effects has been hindered because of the lack of a specific ATP-receptor antagonist. Recently, however, 2-2′pyridylisatogen (PIT) has been shown to block the relaxation of guinea-pig isolated taenia caecum induced by ATP without preventing the inhibitory effects of noradrenaline or isoprenaline (Hooper, Spedding, Sweetman & Weetman, 1974; Spedding, Sweetman & Weetman, 1975). This antagonist has now been used to analyse the action of ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine on the isolated taenia caecum preparation of the guinea-pig. The results provide evidence that there are two or more receptors for the inhibitory effects of the purine bases.

Methods

Taenia caeci preparations were obtained from female guinea-pigs in the weight range 250–600 grams. The preparations were suspended in 10 ml isolated organ baths filled with McEwen's solution (McEwen, 1956) maintained at 35 ± 1 °C and gassed with 95% O_2 and 5% CO_2. After an equilibration period of 30 min, responses were recorded isotonically on a smoked drum (magnification 1 : 4, load 1.5 grams).

Cumulative concentration-response curves (Van Rossum, 1963) for the purine bases and noradrenaline to relax the preparations were determined at 20 min intervals. Each dose of drug was allowed to produce its full effect (5–40 s contact) before the concentration of the drug in the bath was increased. Preparations with low tone, i.e. those that did not contract in the isolated organ bath so that they were 25% or less of their fully relaxed length, were not used.

The initial concentration-response curve for each agonist was disregarded. Subsequent concentration-response curves were measured and the EC_{50} (the concentration producing a 50% maximal relaxation) and slope (ratio of the concentrations producing 80% and 20% maximal effect; Stephenson, 1956) were determined. Dose-ratios were estimated as the ratio of the EC_{50} after and before the modifying drug.

In one series of experiments, attempts were made to desensitize the taenia to ATP and adenosine by including one of the bases in the bathing medium. After 30 min exposure to the desensitizing agent (2 mM), cumulative concentration-response curves for ATP, ADP, AMP, adenosine and noradrenaline were obtained.

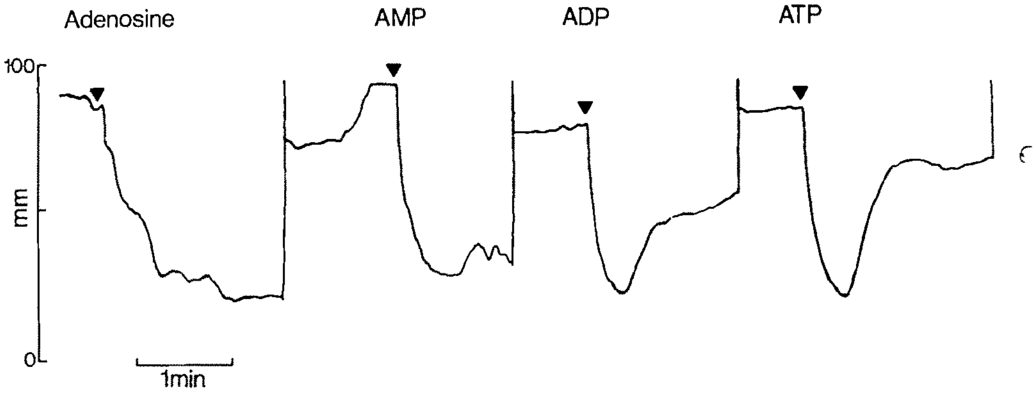

Figure 1 The effects of adenosine (400 μM), adenosine 5′-monophosphate (AMP, 120 μM), adenosine 5′-diphosphate (ADP, 40 μM) and adenosine 5′-triphosphate (ATP, 40 μM) on the taenia. Responses were obtained at 10 min intervals. Zero on the vertical calibration bar represents the maximum relaxation of the preparation to ATP.

Values in the text refer to mean ± s.e. mean. Differences in means were determined by Student's *t*-test, after checking the homogeneity of the variances (Snedecor & Cochran, 1967). Correlation coefficients were determined as described by Daniel (1974) and regression analysis by the method of Bailey (1959).

Drugs

PIT was synthesized by Dr M. Hooper of this department. The other drugs used were: adenosine 5′-triphosphate disodium salt (BDH), adenosine 5′-diphosphate disodium salt (Sigma), adenosine 5′-monophosphoric acid (BDH), adenosine (BDH); (−)-ascorbic acid and carbachol chloride (BDH); dipyridamole (Boehringer); (—)-noradrenaline bitartrate (Koch-Light). Solutions of ATP, ADP and AMP were adjusted to pH 7 with 1 M NaOH. Adenosine was dissolved in 1 M HCl and adjusted to pH 5 with 1 M NaOH. Dipyridamole was dissolved in the minimum volume of 1 M HCl. Noradrenaline was protected from oxidation by the inclusion of approximately 100 μg/ml of (−)-ascorbic acid in each dilution. The composition of the McEwen's (1956) solution was as follows (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11.1 and sucrose 13.2.

Results

Responses of the taenia to the purine bases

ATP, ADP, AMP and adenosine relaxed the taenia in a concentration-dependent manner. However, there were differences in the time to peak relaxation, ATP and ADP causing a rapid relaxation of the smooth muscle, whereas the effects of adenosine and AMP developed more slowly (within 4–40 s of dosing, as opposed to 2–14 s for ATP and ADP, see Figure 1). It was also possible to subdivide the bases into two groups on the basis of the ability of the muscle partially to regain tone whilst the relaxant drug was still in contact with the preparation (Figure 1). In the presence of ATP and ADP a partial regaining of tone was consistently observed, adenosine and AMP exhibiting this effect much less frequently (Table 1), and when it occurred it was much smaller than that following ATP or ADP.

Concentration-response curves for each of the bases were determined, the tissue being dosed cumulatively. The maximum intensity of action obtained with all the purines was similar (< 10% variation, *n* = 5), presumably because the smooth muscle was fully relaxed. The affinity of the drugs for the tissue (EC_{50}), the frequency of occurrence of the secondary contractions, and the slopes of the curves are shown in Table 1.

Effect of 2,2′-pyridylisatogen

PIT (50 μM for 30 min) relaxed the taenia and so carbachol (0.05–1.0 μM) was used to restore the tone of the preparation to within 20% of the control level. In the presence of this cholinomimetic agent PIT is more active (× 5) as an antagonist of ATP (Spedding *et al.*, 1975). In the present experiments, PIT (50 μM for 30 min) markedly antagonized the relaxant effects of ATP and ADP (dose-ratios 46 and 29), but the dose-ratios for AMP (5.0) and adenosine (2.0) indicated a low level of antagonism, being similar to that for noradrenaline (2.4; see Figure 2).

Although it was clear that PIT blocked the effects of ATP and ADP without greatly reducing the

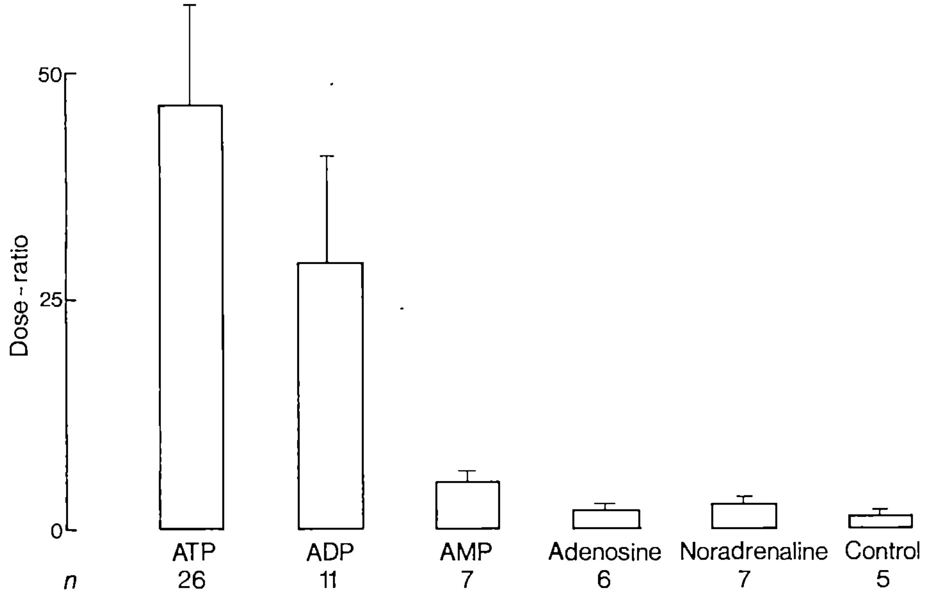

Figure 2 Effects of 2-2′pyridylisatogen tosylate (PIT; 50 μM for 30 min) on the relaxations of the taenia induced by drugs. Carbachol (0.05–1.0 μM) was used after PIT to recontract the tissue to within 20% of the original level. Control experiments in which cumulative concentration-response curves to adenosine 5′-triphosphate (ATP) were obtained when PIT was omitted are also shown. The vertical bars represent the s.e. mean. Note that only the effects of ATP and adenosine 5′-diphosphate (ADP) are antagonized, and that these dose-ratios are not significantly different ($P > 0.05$).

sensitivity of the tissue to AMP and adenosine, the magnitude of the antagonism, particularly of ADP, was not consistent. Preparations that were particularly sensitive to ADP were the most susceptible to the antagonistic action of PIT. The relationship between the effectiveness of PIT and the sensitivity of the preparations to ADP is shown in Figure 3. Analysis of the data in Figure 3 showed a significant. negative correlation between the EC$_{50}$ for ADP and the dose-ratio for PIT, the highest value for the correlation coefficient occurring when log$_{10}$ EC$_{50}$ was compared with the dose-ratio (Table 2). A similar correlation was found following the analysis of the results with ATP: the log$_{10}$ EC$_{50}$ was significantly correlated with the dose-ratio ($r = -0.67$, $P < 0.01$, $n = 19$).

Effect of dipyridamole

Although the inhibitory effects of adenosine and ATP on the taenia were dissimilar with respect to potency, onset and rate of relaxation, and susceptibility to PIT, it is known that adenosine is taken up into cells at a

Table 1 Concentration-response curve data for purines on the guinea-pig taenia

Derivative	*EC$_{50}$ (± s.e. mean) (μM)*	*Slope (± s.e. mean)*	n	*Proportion of preparations partially regaining tone*
ATP	32 (±9)	26 (±5)	21	72/73
ADP	44 (±9)	29 (±7)	11	19/20
AMP	240 (±70)	10 (±2)	7	10/19
Adenosine	230 (±79)	9 (±2)	6	7/34

The drugs were administered cumulatively, increasing the concentration when the relaxation following the previous dose was complete. The initial curve was not used, and each tissue received only one derivative. EC$_{50}$ is the concentration producing a 50% maximal relaxation. The slopes of the curves have been measured as the ratio of concentrations producing 80% and 20% maximal relaxations. Some additional preparations are included in the values where tone was partially regained.

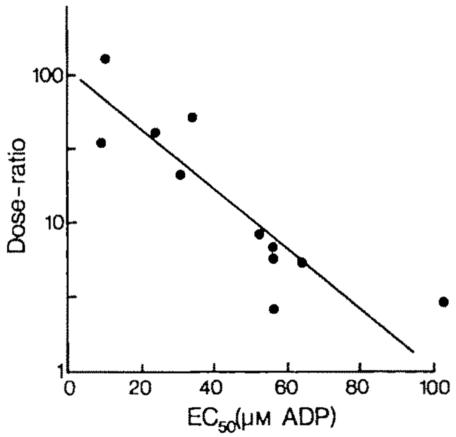

Figure 3 The relationship between the sensitivity of the taenia to adenosine 5'-diphosphate (ADP) and the dose-ratio following incubation with 2-2'pyridylisatogen tosylate (PIT). EC_{50} values were calculated from cumulative concentration-response curves to ADP before incubation with PIT (50 μM for 30 minutes). Carbachol (0.05–0.3 μM) was added after PIT to restore the tone of the preparations to within 20% of their original length. Dose-ratios were calculated from subsequent concentration-response curves. The line is the calculated regression line ($n = 11$).

much faster rate than is ATP. Accordingly, the experiments were repeated in the presence of dipyridamole, a drug known to inhibit adenosine uptake into the tissues (for references see Discussion section).

The results of the experiments using dipyridamole (2 μM) are shown in Figure 4. The dose-ratio for adenosine was now less than 0.1, which indicates that the inhibitory effects of adenosine were potentiated more than tenfold. However, PIT (50 μM for 30 min) in the presence of dipyridamole failed to antagonize the relaxant effects of adenosine. In contrast, the effects of ATP were still antagonized by PIT after dipyridamole.

Desensitization experiments

In preliminary experiments, exposure of the taenia to high concentrations of ATP (2 mM) reduced the sensitivity of the preparations to subsequent additions of both ATP and adenosine, although these effects were variable. In contrast, high concentrations of adenosine caused a desensitization to adenosine but not to ATP. Consequently, a series of concentration-response curves to ATP, ADP, AMP, adenosine and noradrenaline were obtained in the presence of adenosine (2 mM) in the bathing fluid. The EC_{50} values from these experiments are compared to those obtained with normal bathing fluid in Table 3. Adenosine was the only drug whose effects were significantly reduced by the inclusion of adenosine in the medium.

Discussion

This paper contains evidence that ATP and adenosine act on different receptors, confirming the findings of Satchell, Lynch, Bourke & Burnstock (1972) that the nature of the response of the taenia to the two drugs is different. The dissimilar nature of the responses to the two bases was confirmed when PIT was found to be an effective antagonist against ATP but not adenosine-induced relaxations of the taenia. Finally, it was possible to desensitize the taenia to adenosine by including this base in the bathing medium, a procedure which increased the effectiveness of ATP.

The action of ADP resembled that of ATP in the nature of the response, in the blocking action of PIT, and in the desensitization experiments. However, the potency of PIT against ADP varied considerably. This was accounted for when the effectiveness of the

Table 2 Correlation between the sensitivity of the taenia to adenosine 5'-diphosphate (ADP) and the potency of 2-2'pyridylisatogen tosylate (PIT)

Measure of sensitivity to ADP	*Measure of potency of PIT*	r	P
EC_{50}	dose-ratio	−0.72	<0.05
$\log_{10}EC_{50}$	dose-ratio	−0.89	<0.001
EC_{50}	$\log_{10}$dose-ratio	−0.88	<0.001
$\log_{10}EC_{50}$	$\log_{10}$dose-ratio	−0.54	>0.05

ADP was administered cumulatively, increasing the concentration when the relaxation following the previous dose was complete. The initial curve was not used. EC_{50} is the concentration producing 50% maximal relaxation. The dose-ratio represents the EC_{50} after PIT (50 μM for 30 min) divided by the control EC_{50} on that preparation ($n = 11$). Carbachol (0.05–0.3 μM) was used after PIT to restore the tone of the preparations to within 20% of the control level.

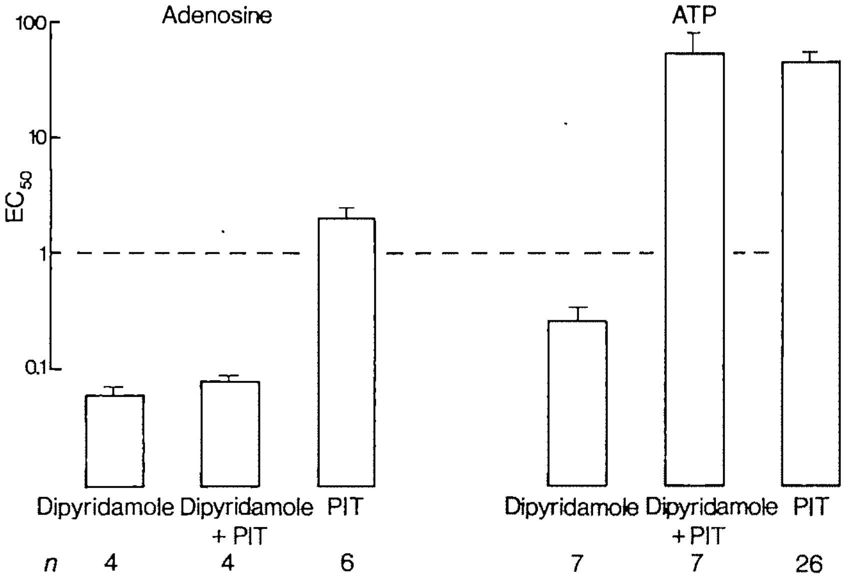

Figure 4 Effects of dipyridamole on the responses of the taenia to adenosine and adenosine 5′-triphosphate (ATP). EC$_{50}$ values were first calculated from cumulative concentration-response curves to adenosine and ATP, then secondly after a 20 min incubation with dipyridamole (2 μM), and finally after incubation with 2-2′pyridylisatogen tosylate (PIT, 50 μM for 30 min) in the presence of dipyridamole. Carbachol (0.05–1.0 μM) was used to restore the tone of the preparations to within 20% of the original length. The results are compared with EC$_{50}$ values obtained after PIT (50 μM for 30 min) in experiments when the dipyridamole was omitted. The vertical bars represent the s.e. mean. Note that PIT failed to block the adenosine-induced relaxations even in the presence of dipyridamole.

antagonist was considered in relation to the sensitivity of the tissue to the nucleotide: the more sensitive the preparation, the greater was the antagonism. A similar relationship existed between ATP and PIT. Such relationships may indicate that ATP, ADP and PIT act at a common site, and that the number of these sites varies from preparation to preparation. However, further studies will be necessary to establish this idea.

Table 3 Effects of including adenosine (2 mM) in the McEwen's solution on the sensitivity of the taenia to purines and noradrenaline

Drug	*EC$_{50}$ (± s.e. mean) Control (μM)*	*In the presence of adenosine (μM)*	*Dose-ratio*	P	
ATP	32 (±9)	7 (±4)	0.2	>0.05	<0.10
ADP	44 (±9)	28 (±11)	0.6	>0.05	
AMP	240 (±70)	340 (±104)	1.4	>0.05	
Adenosine	230 (±79)	1946 (±267)	8.5	<0.001	
Noradrenaline	0.216 (±0.05)	0.197 (±0.07)	0.9	>0.05	

The drugs were administered cumulatively, increasing the concentration when the relaxation following the previous dose was complete. EC$_{50}$ represents the concentration of drug producing 50% maximal relaxation. In this table only, the dose-ratios have been determined by dividing the mean control EC$_{50}$ into the mean value obtained in the presence of adenosine (2 mM for >30 minutes). There were five experiments in the 'In the presence of adenosine' group, and the concentration-response curves were obtained at 20 min intervals, the order of dosing being randomized. The 'control' EC$_{50}$ values have been taken from Table 1. Carbachol (0.05–0.2 μM) was used to restore the tone of the preparations to within 20% of the level at the start of the experiment. Note that adenosine was the only drug to which the taenia was significantly desensitized.

It was thought worthwhile to investigate whether differences in the metabolism and uptake of ATP and adenosine into cells could account for the inability of PIT to block the effects of adenosine. Consequently, the experiments were repeated with dipyridamole in the McEwen's solution. Dipyridamole reduces adenosine uptake, probably by inhibiting the intracellular phosphorylation of adenosine to ATP (Hopkins & Goldie, 1971; Hopkins, 1973; Hulme & Weston, 1974). Thus, when Hulme & Weston exposed rabbit intestine to adenosine in the presence of dipyridamole, the intracellular level of adenosine was normal or slightly elevated, but the amount of ATP was much less than in the control group. In the present experiments, PIT failed to block the inhibitory effects of adenosine even though the dipyridamole treatment increased the sensitivity of the taenia to the base 17-fold. In contrast, PIT was equally effective against ATP in the presence and absence of dipyridamole. Thus the differences between ATP and adenosine were not secondary to their disposition in the tissue.

Attempts to differentiate between the effects of ATP and adenosine by specifically desensitizing the taenia were successful when adenosine was the desensitizing agent, but not when ATP was used. The failure may have been due to metabolism of ATP to adenosine by enzymes in the taenia, and this adenosine could subsequently desensitize the tissue to adenosine as well as to ATP. When adenosine was used as the desensitizing agent, it is unlikely that ATP would be generated extracellularly because the enzymes for this biosynthesis are predominantly intracellular. Weston (1973) found that ATP desensitized rabbit duodenum to both ATP and adenosine, but not to noradrenaline and isoprenaline. However, he also found that adenosine desensitized the receptors to ATP as well as to adenosine. The difference between the rabbit duodenum and the taenia may be due to the methods of desensitization; in particular, in the present study much higher concentrations of the nucleotides were used (2 mM compared with 100 μM in Weston's experiments).

From the present evidence, which includes the nature of the responses, the blockade by PIT and the desensitization studies, it is possible to conclude that ATP and ADP act at one receptor, and that AMP and adenosine relax the taenia by some other process or processes.

We are grateful to our colleague Dr M. Hooper for synthesizing the 2-2'pyridylisatogen tosylate; to Dr R.T. Brittain, Allen & Hanburys Ltd., Ware, Herts., for stimulating discussions throughout the course of this work; to Mr D.W. Snowdon for photographing the figures; and to Boehringer for a gift of the dipyridamole.

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THE EFFECTS OF PENTOBARBITONE AND PETHIDINE ON FOETAL BREATHING MOVEMENTS IN SHEEP

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1 Small doses of pentobarbitone (4 mg/kg i.v.) administered to sheep in the last third of pregnancy had little overt effect on the mothers. In the foetus they caused arrest of breathing movements, an alteration in the character of the electrocorticogram and cardiovascular changes which varied with gestational age.

2 In contrast, relatively large doses of pethidine (100–200 mg) administered to the mother had no consistent effect on normal foetal breathing movements, though they abolished the foetal response to hypercapnia.

3 The results are discussed in relation to foetal sleep state.

Introduction

Episodic breathing movements are normally present 40% of the time in the undisturbed foetal lamb *in utero* (Merlet, Hoerter, Devilleneuve & Tchobroutsky, 1970; Dawes, Fox, Leduc, Liggins & Richards, 1970, 1972). This observation differs from the conclusion by Windle (1941) and Barcroft (1946) that the foetus is apnoeic. It is now known that rapid irregular foetal breathing movements in sheep are associated with rapid-eye-movement sleep and are particularly liable to arrest by adverse conditions such as hypoxaemia. Also moderate doses of chloralose (15 mg/kg, i.v.) or pentobarbitone (15 mg/kg, i.v.) given to the ewe abolish foetal breathing movements (Dawes *et al.*, 1972).

In the present experiments the effects of a lower dose of pentobarbitone (4 mg/kg) on the foetus are described, and are compared with those of pethidine in large doses. A brief account of some of these observations has been given elsewhere (Dawes, 1973a).

Methods

Foetuses of known gestational age carried by ewes of mixed breeds were prepared for chronic experiments under maternal epidural and local anaesthesia (Dawes *et al.*, 1972). Catheters were implanted in a maternal carotid artery and external jugular vein. A low midline incision was made using aseptic and antiseptic precautions; the uterus was opened, the foetal neck was exposed and a carotid artery and the trachea were catheterized. The tracheal catheter was advanced until its tip lay within the thoracic cavity. To record the foetal electrocorticogram biparietal stainless steel screw electrodes were placed in contact with the dura mater 1.8 cm apart on either side of the midline; they were isolated electrically by application of cold cure dental acrylic, and the scalp was closed. A soft vinyl catheter was placed in the amniotic cavity. The uterus was closed, the catheters and leads being brought to the exterior through an incision in the maternal flank. In one ewe both twins were catheterized; otherwise only one of twins, where present, was operated on. A continuous slow infusion (5 ml/day) of heparin (400 units/ml) was used to maintain the patency of the foetal carotid artery catheters. Antibiotics, streptomycin 1 G and benzyl penicillin 1 million units, were given intramuscularly once daily for the first 2 days. The ewes were housed in metabolism cages and fed hay and concentrate.

Continuous records of foetal tracheal, carotid and amniotic pressures were made night-and-day on a Schwarzer polygraph. The foetal heart rate was obtained from the carotid pulse with an instantaneous rate-meter (Devices). The foetal arterial pressure was recorded as the difference between carotid and amniotic pressure using an electronic subtraction circuit. Blood samples for gas analysis were drawn

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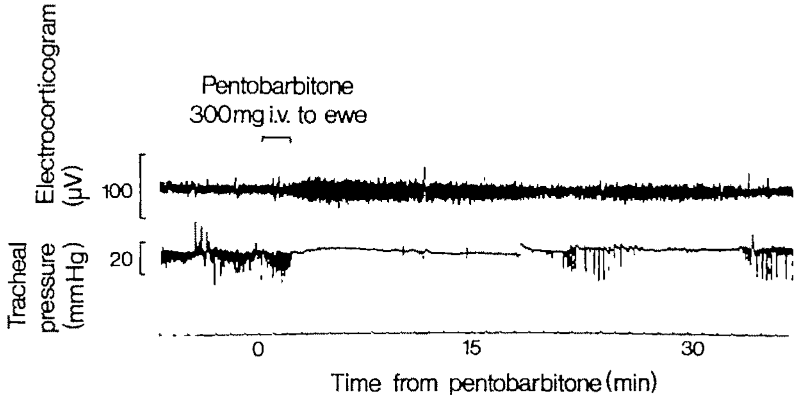

Figure 1 Administration of pentobarbitone (4 mg/kg, i.v. over 2 min) to a pregnant sheep near term caused a change in foetal electrocortical activity (upper record), and arrest of foetal breathing movements.

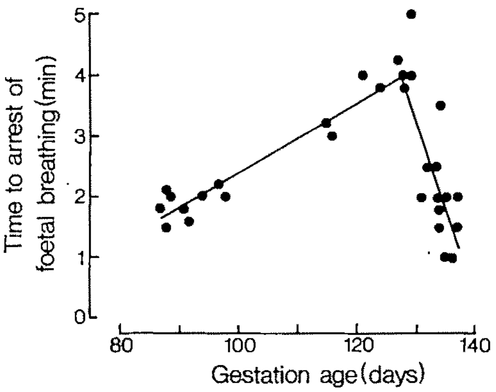

Figure 2 The time to arrest of foetal breathing, after administration of pentobarbitone (4 mg/kg, i.v.) to the mother, varied with gestational age.

into heparinized glass syringes and measured immediately (Radiometer); the results were corrected for the difference between electrode and body temperature, where present. The sheep were allowed to recover and no experiment was performed within 48 h of the operation; thereafter one experiment only was carried out each day. The ewes were weighed before operation and the dose of sodium pentobarbitone (Nembutal, Abbot) was calculated as 4 mg/kg. The pentobarbitone was given slowly to the ewe via the external jugular vein over a 2 min period, during a period of rapid foetal breathing unless stated otherwise, and the catheter was flushed with 10 ml heparinized 0.9% w/v NaCl solution (saline). The start of the injection was taken as time zero. Control measurements were made for the 6 h before the injection. The experiments were performed in the morning or early afternoon to minimize the effects of the diurnal variations in both foetal breathing movements (Boddy, Dawes & Robinson, 1973) and in the time of onset and duration of pentobarbitone action (as in the adult rat; Friedman & Walker, 1968).

Results

Pentobarbitone

The ewe was only lightly sedated by injection of pentobarbitone (4 mg/kg). If standing at the start of the injection she normally remained so; occasionally she continued to eat hay or concentrate. No measurements were made of maternal blood pressure or breathing. On no occasion did the ewe appear to be excited by the dose of pentobarbitone.

Foetal breathing movements. Two types of episodic breathing movements are observed in the sheep foetus, rapid shallow irregular breathing movements at 1–3 Hz (associated with rapid-eye-movement sleep) and slow augmented breaths, described as 'sighs or gasps' (Dawes *et al.*, 1972). When the pentobarbitone was injected during an episode of foetal breathing all breathing movements stopped within 1.0 to 5.0 min (e.g. Figure 1). The time to arrest of foetal breathing was positively correlated with gestational age between 87 and 130 days ($r=0.955$, $P<0.01$) but negatively correlated between 125 and 137 days ($r=-0.69$, $P<0.01$; Figure 2).

After a period of apnoea ranging from 8–31 min foetal breathing movements returned as a series of single inspiratory movements with a rate of 2–3 per minute. The time to the onset of this slow foetal breathing was positively correlated with gestational

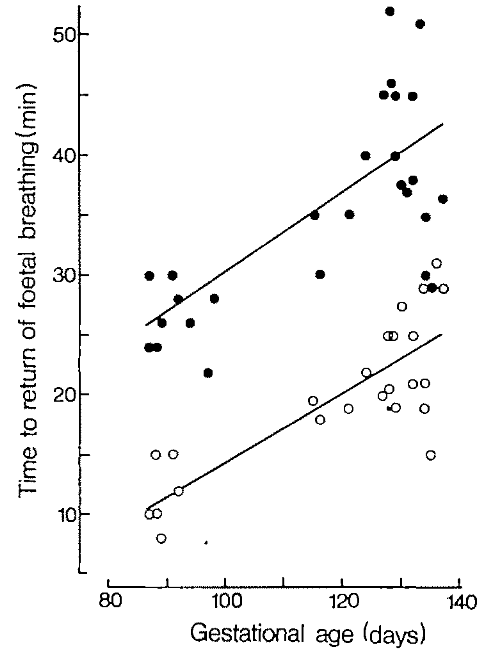

Figure 3 The time to return of slow (O) and of rapid (●) foetal breathing, after maternal administration of pentobarbitone (4 mg/kg, i.v.), varied with gestational age.

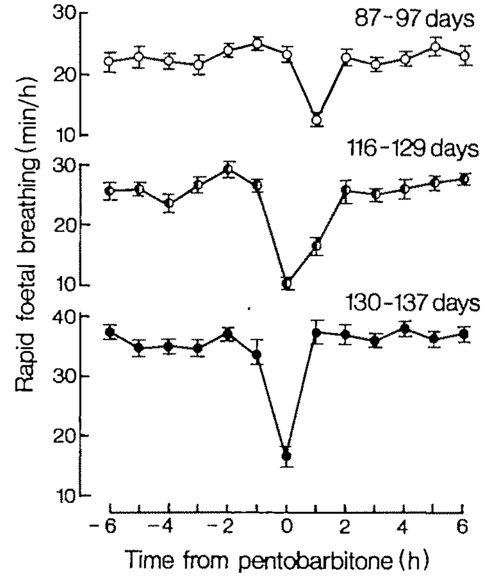

Figure 4 Variations with gestational age in the effects of maternal administration of pentobarbitone (4 mg/kg, i.v.) on the proportion of time during which foetal breathing was present. Note that the proportion varied with gestation before drug administration.

age (Figure 3, open circles). There was then a further period of apnoea before the return of rapid foetal breathing movements. The time to return increased with gestational age (Figure 3, closed circles).

The change in foetal breathing in the 2 h after administration of pentobarbitone varied with gestational age. At 87–97 days (11 experiments, 4 foetuses) the number of minutes of foetal breathing in the first hour after pentobarbitone injection did not differ significantly from that in the 6 h control period (Figure 4). In the second hour it was reduced significantly by 44%. At 116–129 days (7 experiments, 3 foetuses) foetal breathing was reduced significantly from the control values by 60% in the first hour and by 38% in the second hour. In the oldest lambs (130–137 days; 12 experiments, 6 foetuses) foetal breathing movements were only reduced in the first hour following pentobarbitone, by 53%.

Six experiments were done on 3 foetuses of 117–138 days gestation in which pentobarbitone was given to the ewe during foetal apnoea. The breathing movements were reduced from 28.6 ± 0.7 min/h in the 6 h control period to 6 ± 2.0 min in the hour after injection. They were not significantly reduced in the second hour.

Foetal electrocorticogram. The foetal electrocorticogram was recorded in 6 experiments on 4 foetuses between 115 and 134 days. In each lamb soon after the start of a maternal injection of pentobarbitone the electrocortical activity changed gradually from low to high voltage. The rate of change was slower than that observed spontaneously in the transition from rapid-eye-movement to quiet sleep. In the first hour after pentobarbitone administration the incidence of electrocortical low voltage activity was reduced (Figure 5). The number of changes from low to high voltage per hour increased by more than 80% in the 2 h after giving pentobarbitone.

Foetal cardiovascular changes. The effect of pentobarbitone on the foetal heart rate and arterial pressure varied with gestational age. The heart rate in the control period was greater in the younger than in the older foetuses. At 87–97 days there was a small fall in heart rate (Figure 6) after injection of pentobarbitone, which gradually returned towards its initial value.

At 116–129 days the foetal heart rate increased from 158 ± 2 to 181 ± 4 at 10 min (Figure 6); it remained elevated for 45 min and then slowly fell to control values. In the oldest lambs, at 130–137 days, the heart rate rose from 149 ± 3 to 166 ± 5 beats/min for 15 min and then declined.

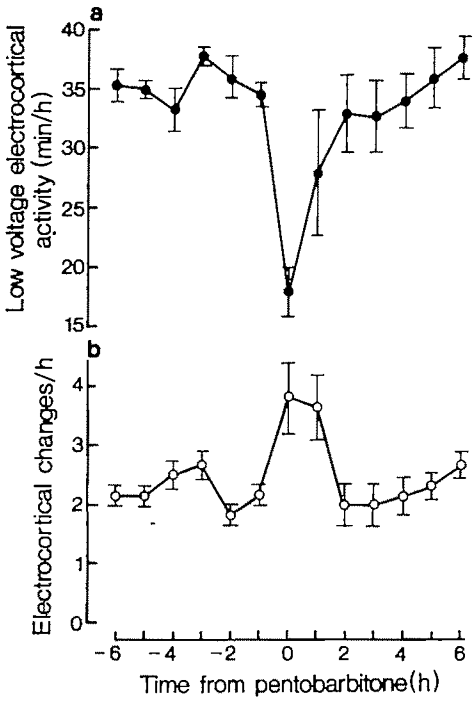

Figure 5 (a) Shows the effect of maternal administration of pentobarbitone (4 mg/kg, i.v.) on the proportion of time during which low voltage foetal electrocortical activity was present. (b) Shows that the number of changes from low to high voltage activity increased as a result of pentobarbitone administration.

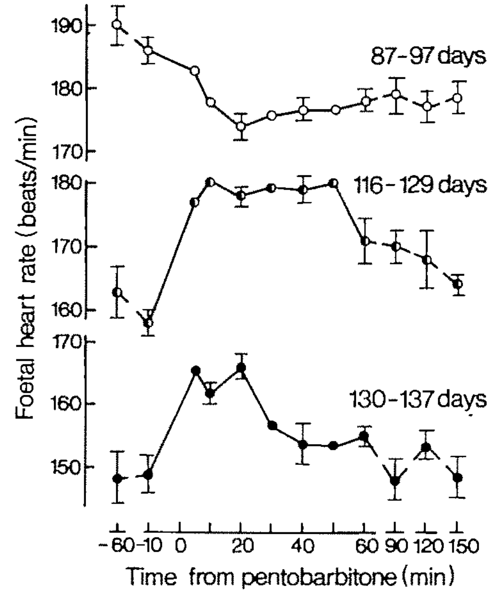

Figure 6 Changes in foetal heart rate, on maternal administration of pentobarbitone (4 mg/kg, i.v.), varied with gestational age. Note the fall in heart rate with age.

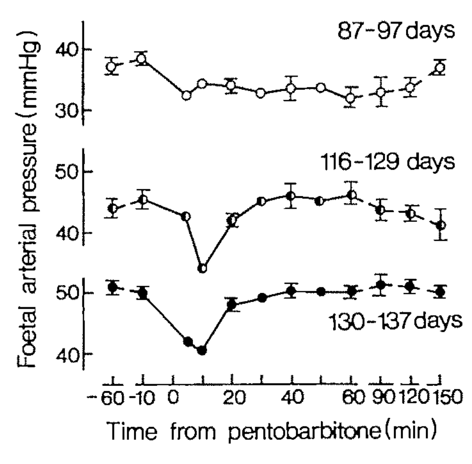

Figure 7 Changes in foetal carotid arterial blood pressure, on maternal administration of pentobarbitone (4 mg/kg, i.v.), varied with gestational age. Note the rise in blood pressure with age.

In the control period the arterial pressure increased with gestational age. In the youngest group of foetuses it fell after pentobarbitone and recovered only very slowly (Figure 7). At 116–129 days, there was a biphasic response, while at 130–137 days there was a brief fall in pressure lasting a bare half hour.

Blood gases and pH. There were barely significant changes in maternal carotid blood gas and pH values after administration of pentobarbitone (4 mg/kg). The foetal carotid $Pa\text{O}_2$ (22 ± 1.6 mmHg), $Pa\text{CO}_2$ (49.9 ± 2.3 mmHg) and pH (7.33 ± 0.007) in 7 experiments on 4 foetuses of 115–136 days gestation were not changed significantly.

Pethidine

Doses of pethidine hydrochloride 100–200 mg were given intramuscularly, intravenously or retrograde via a carotid artery into the ascending aorta in 25 experiments on 13 ewes during the last third of pregnancy. The arterial injections sometimes caused maternal convulsions and were abandoned. There was no consistent effect on foetal breathing movements, blood pressure or heart rate even though maternal administration of pethidine to sheep gives foetal blood

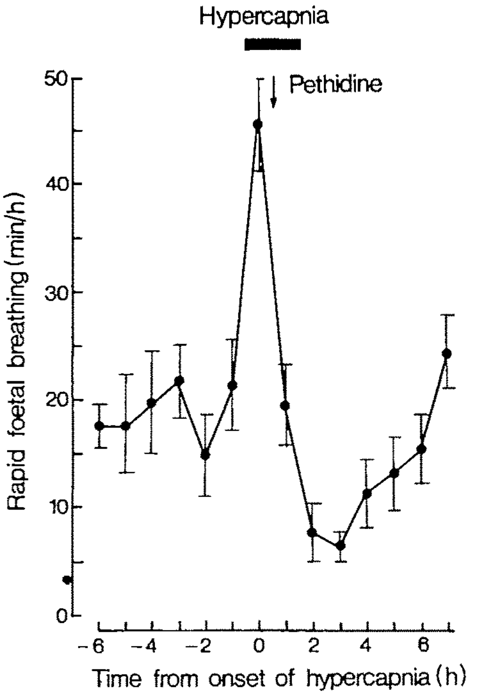

Figure 8 Observations on 5 foetal lambs, showing that maternal administration of pethidine (200 mg, i.v.) abolished the increase in foetal breathing induced by hypercapnia (6% CO_2, 18% O_2 in N_2 given to the ewe).

concentrations which after 10 min are higher than those in the mother (Jenkins, Talbert & Dilts, 1972).

This negative result was surprising. Therefore additional experiments were conducted on 5 foetal lambs in which breathing activity was increased for 2 h by hypercapnia, induced by administration of 6% CO_2 with 18% O_2 in N_2 to the ewe (Boddy *et al.*, 1974). This caused a rise in foetal $Paco_2$ from 42.3 ± 1.6 to 56.7 ± 1.5 mmHg, with no significant change in Pao_2. The proportion of time during which breathing movements were present was more than doubled during the first hour's hypercapnia (Figure 8). Administration of pethidine (200 mg, i.v. to the ewe) at the beginning of the second hour abolished this response. There was subsequently a reduction in the proportion of time during which breathing movements were present, after the end of hypercapnia. In control experiments increased breathing movements were sustained during 2 h hypercapnia.

Discussion

Episodic irregular foetal breathing movements are normally present from early gestation, about 0.27 of full term (40 days in sheep, 11 weeks in man; Boddy & Dawes, 1975). These movements can be affected by the consequences of maternal anaesthesia, by drug action on the foetus and also by the experimental procedure. They are present in foetal lambs only during rapid-eye-movement sleep (Dawes *et al.*, 1972). Procedures which disturb this state, such as restraint after delivery or the acute effects of foetal operation, may prevent the appearance of foetal breathing movements for many hours. They are only present for a normal proportion of the time (~40% in lambs) in chronic preparations in sheep (Dawes *et al.*, 1970; Merlet *et al.*, 1970), or using ultrasound to measure the chest wall movements of intact foetuses *in utero* (Boddy & Robinson, 1971) or acutely on delivery of an intact foetal lamb unrestrained into a warm saline bath under maternal epidural or spinal anaesthesia. Only under such conditions can unambiguous information be obtained as to the effects of drugs on normal foetal breathing movements. Even under otherwise favourable circumstances foetal breathing movements are abolished by a reduction in foetal Po_2 (from 24 to 16 mmHg; Boddy, Dawes, Fisher, Pinter & Robinson, 1974) or by hypoglycaemia. The doses of pentobarbitone used in the present experiments were insufficient to cause a significant change in either Pao_2 or blood glucose. Small doses of barbiturate can cause excitement and catecholamine release. Infusion of catecholamines into a foetal lamb has no effect or increases foetal breathing movements (K. Ritchie; unpublished observations), so the arrest of foetal breathing by pentobarbitone cannot be ascribed to catecholamine release. The abolition of foetal breathing is therefore attributed to a direct effect on the foetus.

Pentobarbitone crosses the sheep's placenta rapidly to give foetal carotid concentrations of about 5 μg/ml plasma 20–40 min after injection of 5 mg/kg into the ewe (Mirkin, 1974). The foetal arterial blood concentration reaches a peak in 20–30 min, whereafter it both exceeds that in the mother and slowly declines over more than an hour. This is in agreement with a theoretical analysis of drug equilibration in the foetus (Dawes, 1973b, c). These data are more consistent with the observed duration of action of pentobarbitone, administered maternally, on the foetal electrocorticogram (Figure 5) than on breathing movements (Figure 1).

In newborn animals breathing spontaneously administration of 4–5 mg/kg pentobarbitone does not normally cause respiratory arrest. Larger quantities are usually required to induce general anaesthesia in newborn rabbits, sheep or rhesus monkeys (Downing, 1960; Dawes, Jacobson, Mott & Shelley, 1960; Dawes, 1968). Mature foetal monkeys delivered under maternal pentobarbitone anaesthesia are readily induced to breathe spontaneously, even after prolonged total asphyxia (Cockburn, Daniel, Dawes, James, Myers, Niemann, Rodriguez de Curet & Ross, 1969). It is therefore evident that irregular episodic foetal breathing movements are unusually susceptible to pentobarbitone, more so than regular maintained breathing after birth. The difference is not explained by the greater susceptibility of very young animals to barbiturates, as measured by sleeping time or the induction of surgical anaesthesia. It is more likely to be due to an effect on the state of sleep. In adult man the dose of pentobarbitone used (4 mg/kg) causes a reduction in rapid-eye-movement sleep activity (Kleitman, 1963).

The effect of pentobarbitone in causing a reduction in the incidence of predominantly low voltage rapid electrocortical activity in foetal lambs can only be examined after 115 days gestation, when electrocortical activity is sufficiently differentiated into its fast and slow components. Bernhard, Kaiser & Kolmodin (1959) found that the lamb's electroencephalogram becomes continuous at 90–100 days gestation, and different foetal sleep states (arousal, quiet and paradoxical sleep) were identified in late pregnancy (Dawes *et al.*, 1970, 1972; Ruckebusch, 1971). In other species of animals and man studied postnatally the sleep-wakefulness pattern can be recognized by changes in behaviour and functions such as breathing before the characteristic electrocortical patterns can be identified (Ellingson, 1972). It may thus be possible to study the effects of barbiturates on the foetal state of arousal or sleep at an earlier stage of cerebral development. The long term effects of barbiturates on the foetal brain are not known, but the evidence that function is necessary for the proper development of structure (e.g. in the development of the visual cortex of the kitten and the somato-sensory region of the mouse) at certain crucial periods is worth noting.

There are complex age-related changes in the times to onset of foetal apnoea and to return of breathing movements (Figures 2 and 3) after maternal administration of pentobarbitone. Interpretation of these differences must be speculative in the absence of information about the time-related changes in foetal plasma pentobarbitone concentrations with gestational age. Data are available for foetal pentobarbitone pharmacokinetics and protein binding in sheep only at 124–136 days gestation as yet (Mirkin, B.L., personal communication). The results are unlikely to be due to changes in the foetal circulation with age (Dawes, 1968; 1973c; Rudolph & Heymann, 1974). They are more likely to be due to changes in foetal hepatic uptake and detoxification and/or to changes in the susceptibility of cerebral tissues to barbiturates with age (as after birth).

Interpretation of changes in foetal heart rate after pentobarbitone administration is complicated by the fact that there are, independently, an association between heart rate and electrocortical activity (Dawes, 1973a). and an inverse correlation between foetal heart rate variability and foetal breathing movements (Dalton, Dawes & Patrick, 1976). The foetal heart rate changes after maternal pentobarbitone administration are not attributable to changes in foetal blood gas values or pH. The baroreceptor mechanisms are increasingly active in the last third of gestation in sheep (Dawes, 1968; Shinebourne, Vapaavuori, Williams, Heymann & Rudolph, 1972) and could explain the heart rate changes in the lambs of 130–137 days (Figure 5) where there is an inverse relation between heart rate and arterial pressure but not otherwise. The initial fall in arterial pressure at all three gestational ages (Figure 6) appears similar to that observed in animals after birth on barbiturate administration, and attributed to systemic vasodilatation. But in this instance pulmonary vasodilatation could also contribute.

It is noticeable that there is a large prolonged rise in heart rate at 116–129 days gestation (Figure 5), associated with restoration of arterial pressure to or above its initial value (Figure 6). This contrasts with the prolonged fall in both heart rate and blood pressure earlier in gestation (87–97 days), at an age when the autonomic control of the foetal circulation is less well developed (Dawes, 1968).

The susceptibility of the foetal lamb to small doses of pentobarbitone, administered to the mother, is in contrast to the absence of any consistent effect with doses of pethidine which, by analogy with those in normal clinical use, are relatively large. Yet these doses of pethidine did abolish the foetal respiratory response to hypercapnia. It is also evident that acute experiments in which the effects of drugs were examined on the foetal circulation under pentobarbitone anaesthesia will require reconsideration, a conclusion arrived at on other grounds by Assali, Brinkman & Nuwayhid (1974) who reported a gross alteration of maternal uteroplacental vasomotor reactivity by pentobarbitone in anaesthetic doses.

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476P FORRESTER, P.A. (introduced by Straughan, D.W.). L-Glutamate excitations on cortical neurones: influence of endogenous neuronal activity and halothane

AN IRREVERSIBLE EFFECT OF LITHIUM ADMINISTRATION TO PATIENTS

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1 Lithium administration to patients leads to a pronounced inhibition (about 90%) of the choline transport system in erythrocytes. The transport system does not recover when ghosts are prepared from the erythrocytes, thereby removing intracellular as well as extracellular lithium.

2 When a patient is taken off lithium, the choline transport in erythrocytes recovers only very slowly over a period of three months, i.e. at about the same rate at which the erythrocytes that had been exposed to lithium are replaced by new cells.

3 It is concluded that therapeutic concentrations of lithium produce an irreversible inhibition of the choline transport system in human erythrocytes.

Introduction

It has recently been shown that the administration of lithium to patients reduces choline uptake into erythrocytes to around 10% of the normal value (Lee, Lingsch, Lyle & Martin, 1974). A very surprising feature of this inhibition is that it is apparently completely independent of the presence of lithium in the external medium. The activity of the choline transport system in erythrocytes from patients on lithium could not be restored to normal by extensive washing and incubation of the cells in lithium-free buffers. There are other observations suggesting that lithium may produce effects that are not readily reversible. The lithium treatment of rats has effects on transport systems in synaptosomes (Baldessarini & York, 1970) and on renal adenyl cyclase (Geisler, Wraae & Olesen, 1972) that are not dependent on the presence of lithium in the incubation media during the *in vitro* measurement of transport or enzyme activity. In both of these papers the authors point out that their results are compatible with an effect of lithium that is not readily reversible, but no efforts were made to remove the lithium that might be trapped in the vesicles that form during tissue homogenization. Baldessarini & York (1970) found in fact that their synaptic vesicles still contain 0.07 mEq/l of lithium. It is therefore conceivable that the persistence of the effects described is the result of the presence of small concentrations of lithium in the vesicles. These results suggest that the therapeutic use of lithium may produce an irreversible inhibition of certain transport processes in man. We have therefore determined whether inhibition of the choline uptake into red cells that is associated with lithium treatment is truly irreversible or a consequence of the presence of lithium inside the cells.

Methods

Erythrocytes from fresh heparinized blood of healthy volunteers and patients were obtained and washed as described previously (Martin, 1972). The patients studied were treated at Fulbourn Hospital, Cambridge for manic depressive disease and the erythrocytes were obtained when blood was taken at the hospital to determine plasma lithium levels.

Choline influx

The procedures for measurement of the influx of [^{14}C]-choline and for calculation of the influx of choline into erythrocytes have been described previously (Martin, 1972; Lee *et al.*, 1974). The rate of choline influx was determined after the cells had been incubated for 10 min at 37°C in a buffer containing 1 μM choline.

Choline efflux from released ghosts

To prepare the ghosts, erythrocytes were washed four times with about five volumes of a solution containing (mM): KCl 105, $MgCl_2$ 30, Tris (pH 7.7 at 25°C) 10, disodium edetate (EDTA) 0.2. After the last wash the cells were packed for 10 min at 1500 *g* and squirted into 70 volumes of stirred ice cold lysing solution containing $MgCl_2$ 2, Tris 10, EDTA 0.2 and [^{14}C]-choline and unlabelled choline to give a concentration of 10 μM.

After the addition of the cells the suspension was allowed to stand for about 5 minutes. Sufficient 3 M KCl was added to restore the tonicity to 320 ideal osmolar and the suspension was incubated at 37°C for 90 min; in some experiments, described in the text, sufficient 2 M LiCl to give 4mM LiCl was added after 30 min of incubation. The ghosts were then washed and the efflux of radioactive choline was followed by taking aliquots of the suspension at 5, 15, 25, 35 and 60 min and measuring the radioactivity in the supernatant; the rate constant for the efflux of choline was calculated from a graph on which the fraction of radioactivity remaining in the ghosts was plotted on a log scale against time (Martin, 1971).

The efflux of choline was always measured in the absence as well as in the presence of 1 mM hemicholinium-3 (HC-3) in the external medium. The HC-3-sensitive efflux was calculated by subtracting the rate constant found in the presence of HC-3 from the rate constant found in its absence.

Lithium determinations

All lithium concentrations given in this paper were determined with a Unicam SP 90 Atomic Absorption Spectrophotometer and standards varying from 2 µM to 2 mM LiCl.

Materials

The sources for the chemicals used were the same as described previously (Martin, 1972). The [methyl-^{14}C]-choline chloride (The Radiochemical Centre, Amersham) had a specific activity of 40–60 mCi/mmol.

Results

The experiments summarized in Table 1 were designed to obtain some direct evidence concerning the relationship between intracellular lithium levels and the pronounced inhibition of choline transport that is observed after several weeks of lithium administration (Lee *et al.*, 1974). Erythrocytes from healthy individuals were incubated for 90 min in buffers containing 4 mM or 8 mM LiCl, resulting in intracellular lithium concentrations similar to those found in patients treated with lithium. Choline uptake from lithium-free buffers was measured after the cells had been carefully washed. The results indicate that the extent to which the influx is inhibited is apparently not related to the concentration of lithium in the intracellular water. The *in vitro* exposure of normal cells to lithium produced about 25% inhibition, irrespective of whether the cells were incubated in 4 mM or 8 mM LiCl. Choline influx into erythrocytes from patients on lithium was only about 15% of the normal value, confirming previous observations (Lee *et al.*, 1974) and suggesting that this second phase of pronounced inhibition is in some way the consequence of prolonged *in vivo* exposure to lithium.

The experiments to be described were designed to examine the effects of removing intracellular lithium as far as possible. Since the lithium-induced inhibition of choline transport is not dependent on the continued presence of lithium in the external medium, or on the concentration of lithium in the intracellular water, washed erythrocytes from patients and healthy individuals were lysed and resealed as described in the section on methods, thus obtaining resealed ghosts that contain [^{14}C]-choline and lithium at only about 1 to 2% of the concentration found in the intact cells. Measurements of the lithium concentrations in the supernatant after the ghosts had been spun down confirmed that virtually all the lithium present in the intact cells had been released.

Unlike intact erythrocytes, resealed ghosts have a significant leak permeability to choline, i.e. some radioactive choline will leave the ghosts even when the carrier mediated choline transport is completely blocked by the presence of 1 mM HC-3 in the external medium (Martin, 1971). The efflux of choline from

Table 1 Choline influx into erythrocytes from patients on lithium and healthy individuals

Origin of blood No. of experiments	*Incubation medium (90 min, 37°C)*	*Intracellular lithium (mEq l cells^{-1})*	*Choline influx (nmol l cells^{-1} min^{-1})*
Patients (11)	Na buffer	0.364 ± 0.053	6.5 ± 1.0
	4 mM Li	0.378 ± 0.061	6.9 ± 1.0
Healthy individuals (12)	Na buffer	—	44 ± 5.0
	4 mM Li	0.218 ± 0.012	33 ± 4.3
Healthy individuals (4)	Na buffer	—	45 ± 5.3
	8 mM Li	0.402 ± 0.021	31 ± 4.6

Aliquots of erythrocyte suspensions were incubated in Na buffer (150 mM NaCl, 10 mM Tris HCl) and 4 mM or 8 mM Li (LiCl added to the Na buffer) as indicated. The concentrations of intracellular lithium were determined after the 90 min incubation period and after washing the cells several times with a lithium free buffer; the haematocrit was determined by measuring the optical density of a suitably diluted aliquot at 540 nm.

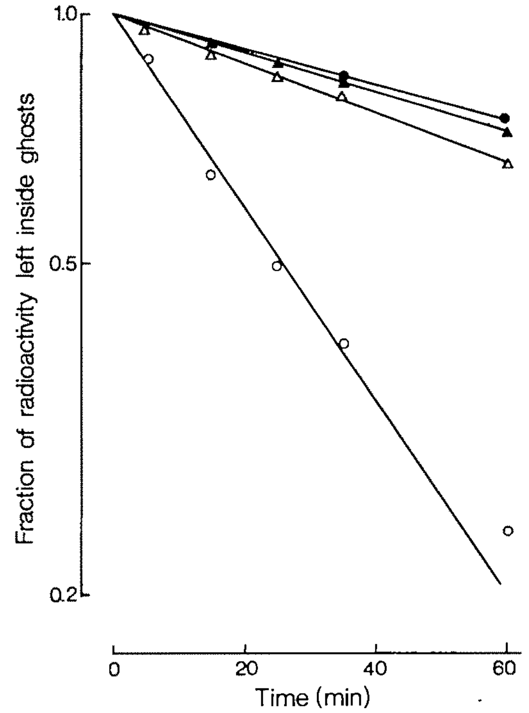

Figure 1 The efflux of [^{14}C]-choline from ghosts prepared from normal erythrocytes (O, ●), and erythrocytes from patients on lithium (△, ▲). The efflux was measured with (●, ▲) and without (O, △) 1 mM hemicholinium-3 in the external medium.

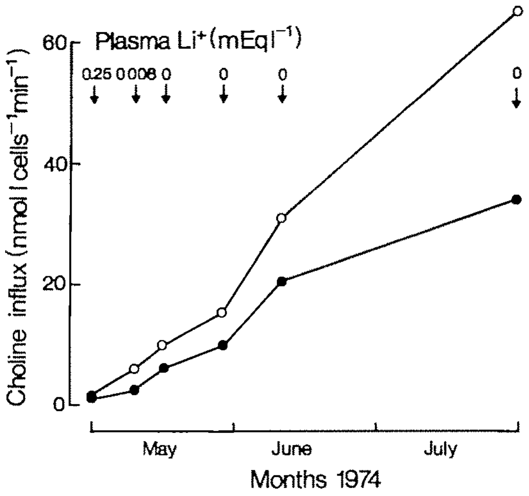

Figure 2 The recovery of choline transport in the erythrocytes of a patient who was taken off lithium on May 1st, 1974. Choline influx was measured after the erythrocytes had been incubated for 90 min in a lithium-free buffer (O) or a buffer containing 4 mM Li (●); the plasma lithium levels were determined using the plasma from the same blood sample.

resealed ghosts is therefore always measured in the presence as well as in the absence of external HC-3; the carrier mediated flux is the difference between the total efflux and the HC-3-insensitive flux. The experimental data shown in Figure 1 were obtained and analysed as described in the section on methods. It is clear that for ghosts prepared from a patient's erythrocytes the rate constant for total choline efflux is only 6.3×10^{-3} min^{-1} and almost the same as that found in the presence of HC-3 (5.02×10^{-3} min^{-1}), indicating that there is very little carrier-mediated choline efflux (1.28×10^{-3} min^{-1}). In contrast to this the data obtained with ghosts from normal blood give a rate constant of 22.1×10^{-3} min^{-1} for the carrier-mediated choline efflux; the HC-3-insensitive fluxes, i.e. the leak permeability to choline is almost identical for the two types of ghosts. Essentially similar results were obtained in all the experiments measuring the efflux of choline from resealed ghosts prepared from the blood of patients on lithium (Table 2). If this inhibition of carrier-mediated choline flux is compared with the reduction of choline influx into the intact cells of the same patients (Table 1) it appears that the removal of intracellular lithium has no effect on the pronounced inhibition of choline transport produced by the administration of lithium. The results of Table 2 also confirm the previous observation (Lee *et al.*, 1974) that there is no obvious correlation between the inhibition of choline transport and the concentrations of lithium in the patient's plasma.

Further evidence indicating that the lithium effect described here is apparently irreversible comes from a study of the recovery of choline transport in a patient taken off lithium (Figure 2). Eight days after the administration of lithium had been stopped the plasma concentration had fallen to about 8 μEq l^{-1} and after a further week no lithium could be detected in the patient's plasma or erythrocytes. In contrast to this the choline transport system in the patient's erythrocytes recovered much more slowly over a period of several weeks; assuming that the life time of human erythrocytes is around 120 days the time course of this recovery might suggest that choline transport returns to normal at the same rate at which old erythrocytes are destroyed and replaced by new ones. When the patient's blood was available for examination for the last time, 3 months after he had been taken off lithium, the value of choline influx was higher than the average found in the normal population but still within normal limits (Askari, 1966; Lee *et al.*, 1974).

As choline transport recovers, *in vitro* exposure of erythrocytes to lithium becomes increasingly effective in producing an inhibition of choline influx (Figure 2). This is again consistent with the idea that the recovery is in fact the result of a gradual replacement of erythrocytes with an irreversibly inhibited choline transport system by normal, newly formed erythrocytes. It is known that *in vitro* exposure to low concentrations of lithium reduces choline influx to between 50 and 70% of the control value in normal cells but has virtually no effect on cells that have already been exposed to lithium *in vivo* (Martin, 1974; Lee *et al.*, 1974).

This evidence suggests very strongly that the pronounced inhibition of choline transport in the erythrocytes of patients treated with lithium is a truly irreversible effect.

Some preliminary experiments were carried out to see whether or not measurable amounts of lithium remain adsorbed to the cell membrane when ghosts are prepared from erythrocytes of patients treated with lithium. After lysing the erythrocytes the membranes were concentrated by centrifugation to about 5% (w/w) and the lithium concentration of this suspension compared with that of the membrane free supernatant. No difference was found; in some experiments the erythrocytes were lysed in 35 volumes of lysing medium, in other experiments in 200 volumes. This allows one to calculate an upper limit for the amount of lithium adsorbed to the membrane. Unfortunately, since the concentration of the membrane suspension used was only 5% (it is difficult to use a significantly higher concentration for technical reasons) and since the lower limit of the spectrophotometer's sensitivity was about 1–2 μM, the value for this upper limit comes to 20–40 μmol of lithium per kg membrane. This is a rather high value; the possibility that significant amounts of lithium remain adsorbed to the cell membrane is therefore not ruled out by these experiments.

Discussion

The purpose of this paper is to show that the administration of lithium to patients can inhibit a physiological function such as choline transport in erythrocytes extensively and irreversibly; the transport system recovers only at the rate at which the affected cells are replaced by new cells.

Baldessarini & York (1970) and Geisler *et al.* (1972) also report lithium effects that persist after the ion has been largely removed. Baldessarini & York (1970) do not discuss possible mechanisms for this

Table 2 Choline efflux from resealed ghosts prepared from erythrocytes of patients on lithium and healthy individuals

Patient	*Plasma lithium (mEq* l^{-1}*)*	*Rate constant for choline efflux,* k *(*10^{-3} min^{-1}*)*
A.F. (M. 53y)	1.09	0.8
M.L. (F. 42y)	1.24	5.5
B.L (M. 25y)	0.77	1.5
R.P. (M. 39y)	0.25	0
J.R. (F. 50y)	1.35	4.1
J.R.	1.27	5.7
D.R. (F. 53y)	0.88	1.4
D.R.	0.80	3.7
D.R.	—	0
R.S. (F. 36y)	0.78	2.0
R.S.	0.67	0
Patients, HC-3-sensitive efflux (mean ± s.e. mean)		2.24 ± 0.65
22 controls, HC-3-sensitive efflux (mean ± s.e. mean)		22.34 ± 2.09
Patients, HC-3-insensitive efflux (mean ± s.e. mean)		4.78 ± 0.38
22 controls, HC-3-insensitive efflux (mean ± s.e. mean)		5.02 ± 0.47

Blood was obtained on 11 separate occasions from a total of 7 patients. Plasma lithium levels were determined and resealed ghosts prepared from the erythrocytes. The rate constant listed for each individual experiment is that of the hemicholinium-3 (HC-3)-sensitive choline efflux. The controls are identical experiments with ghosts prepared from the erythrocytes of healthy individuals.

unexpected persistence, possibly because their experiments do not rule out the possibility that lithium trapped inside the synaptic vesicles might be responsible for it. In the experiments of Geisler *et al.* (1972) there is no indication of trapped lithium and to explain the persistence of the inhibition of adenyl cyclase it has been suggested 'that lithium causes an alteration of some intermediary which itself affects adenyl cyclase' (MacNeil & Jenner, 1975). Nothing is known about the relationship of the rate at which these effects do reverse and the rate at which the affected structures are replaced. The results presented in this paper appear to be the most unequivocal evidence that therapeutic concentrations of lithium can produce an irreversible effect.

The fact that the therapeutic and toxic effects of lithium appear to be reversible does not entitle one to conclude that the basis for these effects must be processes that are reversible on the molecular level. Erythrocytes are very unusual in that their membrane proteins are not continuously removed and replaced. It is now generally accepted that all tissues made up of nucleated cells have a rapid turnover of their membrane proteins (Schimke, 1975). It is therefore conceivable that lithium effects that are irreversible on the molecular level do not appear as such *in vivo* because the structures affected are replaced fairly rapidly.

It is clearly very difficult to envisage how a monovalent cation like lithium, at a concentration of 2 mM, produces this irreversible inhibition of choline transport. Since hexose transport as well as active and passive sodium movement in erythrocytes from patients on lithium are not affected (Lee *et al.*, 1974), one should first consider the possibility that the inhibition of choline transport is a specific effect.

Virtually all biological systems influenced by lithium are physiologically either activated or inhibited by Na^+, K^+, Mg^{2+} or Ca^{2+} and one might therefore expect that the primary cause for any lithium effect is, in the end, the replacement of another cation. There is no indication that choline transport in erythrocytes requires any monovalent or divalent cation; the only site on the transport system known to have an affinity for monovalent cations, including lithium, is the actual transport site (Martin, 1972). It therefore seems reasonable to assume that lithium attacks the transport system by binding to this site. Evidence supporting this assumption is the observation that *in vitro* extracellular choline very effectively protects the carrier against inhibition by lithium (Martin, 1974).

To the erythrocytes this inhibition of choline transport will probably be of no consequence since these cells do not metabolize choline; the choline transport system in their cell membrane appears to be merely a vestige. The relevant question is whether or not a similar inhibition of choline transport occurs in other tissues, especially the central nervous system.

It may seem that an answer to this question could easily be obtained by studying the effects of lithium administration to rats on the various choline transport systems. However, preliminary experiments in our laboratory have shown that the choline transport system in the erythrocytes of rats and rabbits is not affected by the prolonged administration of lithium to these animals. Similarly, while choline transport in human erythrocytes is inhibited by incubation of the cells with lithium *in vitro* (Martin, 1974), this effect cannot be obtained with erythrocytes from rats or rabbits. This suggests that the lithium effect on choline transport described in this paper might be species specific.

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THE ROLE OF BOUND CALCIUM IN SUPERSENSITIVITY INDUCED BY COCAINE

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1 Noradrenaline caused a small contraction of the cat isolated spleen strip bathed in a calcium-free solution; this contraction was greatly potentiated by cocaine. This potentiation was also present in isolated spleen strips where noradrenaline stores were depleted by reserpine. The maximum response of the spleen strip to noradrenaline in the absence of extracellular calcium was also increased by cocaine.

2 The disodium salt of ethylenediaminetetraacetic acid (Na-EDTA) but not Ca-EDTA antagonized the potentiation of the response to noradrenaline by cocaine in a calcium-free solution, and greatly reduced the magnitude of subsequent responses to noradrenaline and cocaine.

3 Strontium caused equivalent contractions of normal and reserpine-treated spleen strips bathed in a calcium-free solution. These responses were potentiated by cocaine.

4 Histamine caused a small contraction of the isolated spleen strip bathed in a Ca-free solution. Cocaine failed to potentiate these very small histamine contractions, but did potentiate the contraction of these same strips in response to noradrenaline.

5 It is concluded that the potentiation of the response of the isolated spleen strip to noradrenaline by cocaine in the absence of extracellular calcium is due to a mechanism other than decreased neuronal uptake of noradrenaline. It is suggested that cocaine makes a bound store of calcium more available to promote contraction of the spleen strip by noradrenaline.

Introduction

A number of recent studies suggest that the supersensitivity induced by cocaine is not entirely due to the blockade of neuronal uptake (Maxwell, Wastila & Eckhardt, 1966; Bevan & Verity, 1967; Kasuya & Goto, 1968; Reiffenstein, 1968; Kalsner & Nickerson, 1969; Varma & McCullough, 1969; Davidson & Innes, 1970; Innes & Karr, 1971; Shibata, Kuchii, Hattori & Fujiwara, 1974). Various other possible mechanisms have been considered. For example, increase of affinity of the receptors for the agonist (Maxwell *et al.*, 1966; Reiffenstein, 1968; Innes & Karr, 1971) has been proposed, but Innes & Mailhot (1973) showed that cocaine does not increase the affinity of α-adrenoceptors for noradrenaline. It has also been suggested that the supersensitivity induced by cocaine may be due to an alteration in the utilization of calcium for contraction (Greenberg & Innes, 1968; Kasuya & Goto, 1968; Greenberg & Long, 1971; Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971). We have therefore studied the effect of cocaine on the utilization of bound calcium in the contraction of cat spleen strips stimulated by noradrenaline.

Methods

Cats (0.3–3 kg) of either sex were killed by a blow on the head. The spleen was removed and strips 20 mm long and 4–5 mm wide were cut from the edge. Each strip was suspended in a muscle bath containing 10 ml of bathing solution kept at 37°C, and bubbled with a gas mixture of 95% O_2 and 5% CO_2. Isotonic contractions against 1 g tension were recorded on a kymograph at ten times lever magnification. The strips were allowed to equilibrate in the bath for 1 h before drugs were added. The bathing fluid was replaced every 15 min except when drugs were present in the bath.

Bathing solutions were made with distilled demineralized water. Standard Krebs-Henseleit solution was of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose, 11.0. In experiments where the tissues were to be bathed in a medium without calcium, a 'Ca-free' solution was prepared by omission of $CaCl_2$ from the standard Krebs-Henseleit formula. To deplete the preparation of calcium the chelating agent disodium ethylenediaminetetraacetic acid (Na-EDTA), 0.3 mM, was added to the Ca-free solution; this was termed a 'zero-Ca EDTA' solution.

Stock solutions of all drugs were made in concentrations of 1, 5, or 10 mg/ml and stored at 4°C.

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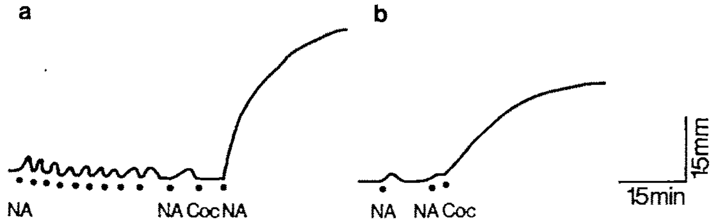

Figure 1 Potentiation of noradrenaline by cocaine in isolated spleen strip of the cat. Tissue calcium of two strips from the same spleen was reduced in Ca-free solution by repeated exposure to noradrenaline (NA, repeated at dots), 1 µg/ml, in a zero-Ca EDTA solution. (a) Cocaine (Coc), 10 µg/ml, was given 5 min before noradrenaline (NA), 1 µg/ml. (b) Cocaine (Coc), 10 µg/ml was given at the peak of the response to noradrenaline (NA), 1 µg/ml.

(–)-Noradrenaline bitartrate and tyramine hydrochloride were dissolved in 0.01 N HCl. Cocaine hydrochloride, EDTA disodium and dicalcium salts, strontium chloride, and calcium chloride were dissolved in distilled demineralized water. A stock solution of reserpine, 5 mg/ml, was made by dissolving 100 mg reserpine in 2 ml glacial acetic acid, 2.5 ml propylene glycol, 2.5 ml 95% ethanol, and sufficient water to make 20 ml. Cats were given 1 mg/kg reserpine intraperitoneally 24 h before an experiment. Spleen strips were shown to be depleted of noradrenaline by the loss of response to tyramine, 30 µg/ml. All drugs were appropriately diluted with 0.9% w/v NaCl solution (saline) on the day of use; with the catecholamines, 0.01 N HCl was added to delay oxidation. Drug concentrations are expressed as final concentration in the muscle bath in µg/ml, except calcium and strontium which are expressed in molar concentrations. Doses of noradrenaline and tyramine are expressed as free base, all other drugs as the salt.

Contractions of the spleen strip were measured at the peak response after they had reached a steady state. Relaxation was calculated as the difference between the magnitude of the peak contraction in response to an agonist and the contraction remaining after 30 min of exposure to an antagonist. When comparisons were made between spleen strips from the same cat, or within the same spleen strip the results were analysed by the *t* test for paired observations (Goldstein, 1964). Observations from different spleens were compared by Student's *t* test.

Results

Potentiation of noradrenaline by cocaine in a calcium-free solution

In these and subsequent experiments a Ca-free solution was used to eliminate an extracellular source of calcium, and zero-Ca EDTA solution was used to promote the removal of interstitial or loosely bound calcium.

Cocaine (10 µg/ml) potentiated the response to noradrenaline (1 µg/ml) in Ca-free solution. Spleen strips were first equilibrated in a zero-Ca EDTA solution for 60 minutes. Tissue calcium was then reduced by repeated additions of noradrenaline until the contraction obtained was small. The tissues were then bathed in Ca-free solution for 30 min to remove the EDTA. Noradrenaline was again added to the bath; a contraction was obtained; the bath was washed out and the tissue was allowed to relax. The strip was then either treated with cocaine for 5 min, after which noradrenaline was added to the bath in the presence of cocaine (Figure 1a), or cocaine was added after the response to noradrenaline had reached a plateau (Figure 1b).

In 36 strips from 32 cats pretreatment with cocaine significantly increased the height of the noradrenaline contraction by 9.9 ± 1.3 mm. In another 47 strips from 31 cats the superaddition of cocaine significantly increased the height of the noradrenaline contraction by 9.1 ± 1.2 mm. Many of these strips received further treatment, which is described in subsequent experiments.

Potentiation of the maximum contraction to noradrenaline by cocaine in calcium-free solution

Experiments were done on 8 strips, two from each of 4 cats. Strips were first bathed in a zero-Ca EDTA solution for 2 h to remove tissue calcium. The bathing solution was then changed to a Ca-free solution to remove the EDTA. Cumulative doses of noradrenaline (1 to 300 µg/ml) were added to the bath until a maximum contraction was obtained (Figure 2). Cocaine (10 µg/ml) was added to the bath when noradrenaline caused no further contraction. Without cocaine the maximum height of the noradrenaline contraction was 21.8 ± 5.4 mm, reached with concentrations of 30 or 100 µg/ml. Cocaine significantly

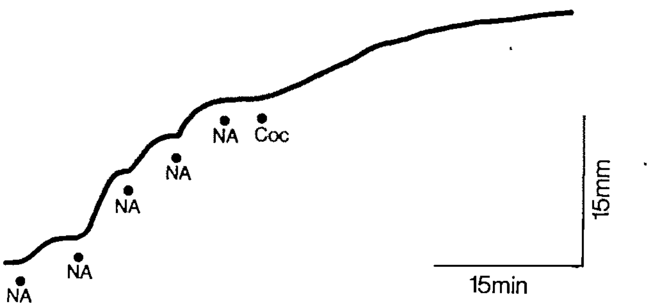

Figure 2 Potentiation of the maximum contraction to noradrenaline by cocaine in isolated spleen strip of cat in Ca-free solution. Strip was kept in zero-Ca EDTA solution for 2 h, then tested in Ca-free solution with noradrenaline (NA) in cumulative concentrations of 1, 3, 10, 30, 100 μg/ml, followed by cocaine (Coc), 10 μg/ml.

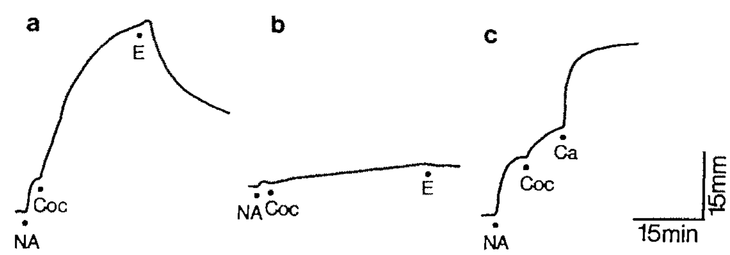

Figure 3 The effect of repeated administration of Na-EDTA on the potentiation of noradrenaline by cocaine in a single isolated spleen strip of cat in Ca-free solution. At NA, noradrenaline 1 μg/ml, at Coc, cocaine 10 μg/ml, at E, Na-EDTA 1 mg/ml and at Ca, calcium 2.5 mM. Between (a) and (b) the strip relaxed for 30 minutes. Between (b) and (c) the strip was kept in Krebs-Henseleit solution for 60 min, which was replaced by Ca-free solution just before the test with noradrenaline.

increased the height of the maximum noradrenaline contraction by 3.5 ± 1.1 mm ($P < 0.02$).

The effect of Na-EDTA on the potentiation of noradrenaline by cocaine in a calcium-free solution

The above results indicate that cocaine may enable noradrenaline to utilize membrane or intracellular bound calcium more effectively. Experiments were therefore done to see if Na-EDTA would alter the potentiation of noradrenaline by cocaine in a Ca-free solution, possibly by chelating this calcium once it was released from its binding site.

Eight spleen strips each from a different cat were first bathed in a zero-Ca EDTA solution for 2 h to reduce tissue calcium. The bathing solution was then changed to Ca-free and kept in this solution for 30 min to remove the EDTA. Noradrenaline (1 μg/ml) caused a small contraction of 6.8 ± 1.0 mm. The addition of cocaine (10 μg/ml) to the bath significantly increased the height of this contraction by 20.6 ± 4.0 mm. The further addition of Na-EDTA (1 mg/ml) caused the strips to relax by 15.7 ± 2.8 mm, thus reducing the potentiation caused by cocaine (Figure 3a). After 30 min in Ca-free solution noradrenaline, cocaine and Na-EDTA were tested again and the noradrenaline contraction, the potentiation by cocaine, and the relaxation by Na-EDTA were all reduced, being 2.8 ± 0.9, 2.1 ± 0.4, and 1.1 ± 0.3 mm respectively (Figure 3b). The preparation was then bathed in standard Krebs-Henseleit solution to replace some of the tissue calcium. After 1 h the bathing solution was changed back to Ca-free. After this replacement of calcium the same dose of noradrenaline caused a larger contraction, 13.8 ± 3.5 mm; this was increased by 7.9 ± 3.0 mm by cocaine (Figure 3c). The addition of 2.5 mM calcium caused a further contraction of 10.6 ± 2.1 mm.

The effect of Ca-EDTA on the potentiation of noradrenaline by cocaine in a Ca-free solution

To exclude the possibility that the inhibition of the cocaine potentiation of noradrenaline by EDTA might be a direct effect of EDTA rather than by chelation of calcium, four experiments were done, each on 2 strips

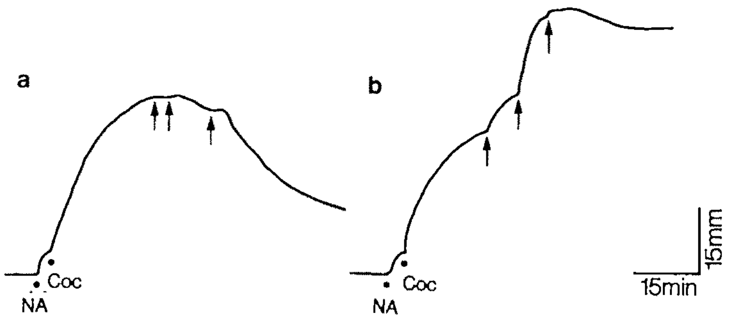

Figure 4 The effect of Na-EDTA and Ca-EDTA on the potentiation of noradrenaline by cocaine in isolated spleen strip of cat in Ca-free solution. Two strips from the same spleen were exposed to noradrenaline (NA), 1 μg/ml, followed by cocaine (Coc), 10 μg/ml, and then exposed at arrows to either Na-EDTA or Ca-EDTA in cumulative concentrations of 10, 100, 1000 μg/ml. (a) Treated with Na-EDTA; (b) treated with Ca-EDTA.

cut from the same spleen. One strip was tested with Na-EDTA and the other with Ca-EDTA (Figure 4). Both strips were first bathed in a zero-Ca EDTA solution for 2 h to reduce the tissue calcium. The bathing solution was then changed to Ca-free, to remove the Na-EDTA. Noradrenaline (1 μg/ml) caused a small contraction which was potentiated by the addition of cocaine (10 μg/ml) to the bath. The strips were then exposed to either Ca-EDTA or Na-EDTA in cumulative concentrations of 10 μg, 100 μg, and 1 mg/ml. All three concentrations of Ca-EDTA substantially increased the already potentiated contraction by 6.5 ± 1.7, 20.8 ± 3.4, 28.0 ± 3.4 mm respectively. The first two concentrations of Na-EDTA did not change the noradrenaline cocaine contraction, but the largest concentration substantially relaxed the strip by 20.0 ± 6.4 mm.

Four experiments were done on strips with normal Krebs-Henseleit solution. Ca-EDTA or Na-EDTA (1 mg/ml) alone had no effect, but Na-EDTA significantly reduced the contraction to noradrenaline (1 μg/ml) by 3.5 ± 0.9 mm ($P < 0.05$) whereas Ca-EDTA had no effect.

Spleen strips taken from four cats pretreated with reserpine, 1 mg/kg, were bathed in a Ca-free solution. Cocaine, 10 μg/ml, potentiated the response to noradrenaline 1 and 10 μg/ml. This potentiation was inhibited by Na-EDTA, 1 mg/ml.

Potentiation of the strontium-induced contraction by cocaine in a calcium-free solution

Strontium has been shown to cause contraction of smooth muscle (Daniel, 1963) and can substitute for calcium in the contraction of smooth muscle (Daniel, 1965; Hudgins, 1969; Toda, 1972). Experiments were done to see if strontium would contract the isolated spleen strip in the absence of extracellular calcium, and whether cocaine would potentiate this response.

Eight spleen strips from 4 normal cats and 8 spleen strips from 4 cats given reserpine (1 mg/kg, 24 h previously) were used; each strip served as its own control. Calcium was removed by exposure of the normal strips to noradrenaline (1 μg/ml) in the presence of a zero-Ca EDTA solution, and by bathing the reserpine-treated tissues in a zero-Ca EDTA solution for 2 hours. All strips were then kept in Ca-free solution. The strips from reserpine-treated cats were first tested with tyramine (30 μg/ml) for completeness of catecholamine depletion; no contraction occurred. Strontium added to these strips caused contraction. Four normal and four reserpine-treated strips were exposed to cumulative concentrations of 0.16 to 12.0 mM strontium until the maximum contraction was obtained. These strips were then re-exposed to the same strontium concentrations in the presence of cocaine (10 μg/ml). In four additional reserpine-treated and 4 normal strips from the same cats the order of treatment was reversed; the tissues were treated with cocaine first followed by the control test of strontium 150 min after the cocaine had been removed. Strontium caused a maximum contraction of 9.1 ± 3.3 and 7.5 ± 1.5 mm in the normal and reserpine-treated strips respectively. In the presence of cocaine the contraction of the same spleen strips was 29.0 ± 4.7 and 15.5 ± 1.9 mm respectively.

Potentiation of histamine by cocaine in a calcium-free solution

Experiments were done with 16 strips from 8 cats. The strips were first equilibrated in a zero-Ca EDTA solution for 1 hour. Tissue calcium was then reduced by repeated addition of histamine to the bath until a contraction of no more than 1 or 2 mm was obtained. The tissues were then bathed in a Ca-free solution to remove the EDTA. Histamine (1 μg/ml) was then

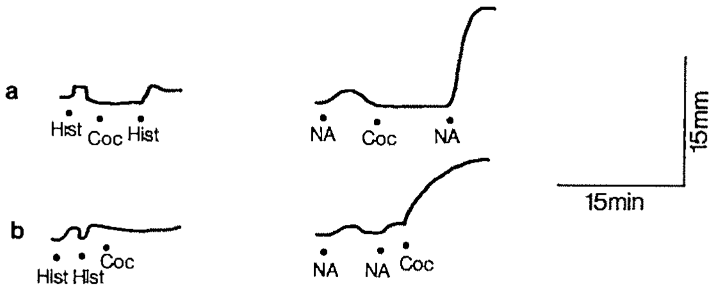

Figure 5 The effect of cocaine on the histamine-induced contraction of the isolated spleen strip of cat in Ca-free solution; strips (a) and (b) exposed to histamine (Hist), 1 μg/ml, before and in the presence of cocaine (Coc), 30 μg/ml; 150 min later both strips (a) and (b) exposed to noradrenaline (NA), 1 μg/ml before and in the presence of cocaine (Coc), 30 μg/ml.

tested in four strips 5 min after cocaine (30 μg/ml) had been added. In another 12 strips cocaine (30 μg/ml) was added after the contraction due to histamine (1 μg/ml) had reached its maximum. Cocaine did not potentiate the contraction in either case (Figure 5). However, 150 min later cocaine was still able to potentiate the response to noradrenaline (1 μg/ml).

Discussion

The results suggest that potentiation of noradrenaline by cocaine in spleen strips in the absence of calcium is probably due to a mechanism other than decreased neuronal uptake of noradrenaline. Although other possibilities cannot be excluded, the most likely explanation is that cocaine in some way makes a bound store of calcium more available for contraction.

Maengwyn-Davies & Koppanyi (1966) found that large concentrations of cocaine (1 mg/ml) caused the contraction of the rabbit isolated aortic strip by the release of endogenous catecholamines. Daniel & Wolowyk (1966) showed that very large concentrations of cocaine caused the contraction of the isolated uterus, by a direct action on the smooth muscle. However, in our experiments, where the maximum concentration of cocaine used was 10 μg/ml, much less than in the above experiments on aortic strip and uterus, cocaine never caused contraction of the spleen strip bathed in a calcium-free solution, but did potentiate the small contractions caused by noradrenaline. Depletion of the catecholamine stores by reserpine did not affect this potentiation by cocaine. Kirpekar & Wakade (1968) showed that extracellular calcium is necessary for the release of noradrenaline from postganglionic sympathetic nerves in the cat spleen. Therefore neither a direct contractile effect of cocaine on the smooth muscle nor release of endogenous catecholamines appears to be responsible for the potentiation by cocaine in the absence of extracellular calcium.

Our finding that cocaine increased the maximum response to noradrenaline in the spleen strip bathed in a calcium-free solution agrees with observations by Barnett, Greenhouse & Taber (1968) and Kasuya & Goto (1968) on rat isolated vas deferens in low calcium solutions. The increased maximum contraction is unlikely to be due to a greater noradrenaline concentration at the receptor as the result of blockade of neuronal uptake by cocaine, because the maximum contraction was obtained with noradrenaline concentrations of 30 μg/ml and 100 μg/ml and further increases in the concentration of noradrenaline did not cause additional contraction, but cocaine did.

Cocaine potentiated the responses to noradrenaline in a calcium-free solution, which were inhibited by EDTA. These results could be accounted for by a more effective release of bound calcium by noradrenaline, or by a more efficient use of the amount of calcium released by noradrenaline. These results are in contrast to the findings of Shibata *et al.* (1971) who found that cocaine did not potentiate the contraction of the rabbit aorta in response to noradrenaline in a Ca-free solution. The reason for this discrepancy might be explained by tissue and species differences, or by the possibility that loosely bound calcium was not removed in Shibata's experiments.

The experiments with strontium provide further evidence for a post-junctional site of action for cocaine. Strontium can substitute for calcium in the contraction of smooth muscle (Sperelakis, 1962; Daniel, 1963; Bohr, 1964; Hudgins, 1969), possibly by displacing bound intracellular calcium (Daniel, 1965; Toda, 1972). Our experiments where pretreatment of the cat with reserpine did not reduce the contraction caused by strontium in spleen strips bathed in calcium-free solution indicate that the contraction was not due to the release of stored noradrenaline. Our results are fully consistent with the hypothesis that strontium causes contraction of the isolated spleen strip by releasing bound calcium, and that this bound calcium is much more easily released in the presence of cocaine. The results therefore suggest a post-junctional and perhaps identical site of action for the effect of cocaine on the contractions due to both noradrenaline and strontium in the calcium-depleted tissue.

Spleen strip contractions due to histamine were greatly reduced by the absence of extracellular calcium, and by loss of tissue calcium from repeated exposure to histamine in the presence of a zero-Ca EDTA solution. Cocaine failed to potentiate these very small histamine contractions, but did potentiate the contraction of these same strips in response to noradrenaline. These results are in agreement with the findings of Hudgins & Weiss (1968) who showed that histamine depends mainly on extracellular and loosely bound calcium for the contraction of smooth muscle, while noradrenaline can utilize a tightly bound calcium store. It is possible that cocaine makes a bound calcium store more available for contraction, and that this calcium is then utilized by noradrenaline and not by histamine.

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INHIBITION OF PERISTALSIS IN GUINEA-PIG ISOLATED ILEUM AND COLON BY DRUGS THAT BLOCK PROSTAGLANDIN SYNTHESIS

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1 Methods of analysing peristaltic activity have been evaluated by the use of recordings of longitudinal and circular muscle activity and of propulsion in whole segments of guinea-pig ileum and colon.

2 Some prostaglandin synthesis inhibitors, and antagonists of prostaglandin action were tested for their suitability for studying the role of prostaglandins in peristalsis. Aspirin was suitable; at 10–200 µg/ml it had little effect on responses of longitudinal muscle strips of the guinea-pig ileum to acetylcholine (ACh), histamine, nicotine or prostaglandin E_2. Indomethacin (1–4 µg/ml) reduced responses to nicotine and prostaglandin E_2. The prostaglandin antagonists polyphloretin phosphate and SC-19220 reduced contractions of ileal longitudinal muscle caused by nerve excitation with either nicotine or transmural stimulation.

3 Aspirin (20–100 µg/ml) or indomethacin (1–4 µg/ml) applied serosally greatly inhibited all aspects of peristalsis in guinea-pig ileum and colon. Inhibition of peristalsis of the ileum by aspirin was antagonized by prostaglandin E_2 and that by indomethacin was removed by prostaglandin $F_{2\alpha}$ or ACh. Inhibition of colonic peristalsis by aspirin was antagonized by prostaglandin E_2 but rarely by ACh, and that by indomethacin by prostaglandin E_1 or E_2. Mucosal application of aspirin had little effect on either ileum or colon but indomethacin caused some inhibition.

4 These results support the supposition that prostaglandins contribute to peristaltic activity.

Introduction

Studies in various species have demonstrated prostaglandin-like material in the gastrointestinal tract which may contribute to muscle tone and activity (see reviews by Bennett & Fleshler, 1970; Bennett, 1972; 1976). The following discussion is restricted to guinea-pig intestine. Prostaglandin E_2- and $F_{2\alpha}$-like materials occur in the ileum (Ambache, Brummer, Rose & Whiting, 1966; and unpublished data). Low concentrations of prostaglandins E_1, E_2, $F_{1\alpha}$ or $F_{2\alpha}$ contract the longitudinal muscle of guinea-pig isolated intestine, apparently in part by direct action on the muscle cells, and partly by stimulating nerves. In the ileum the nerves stimulated are cholinergic irrespective of the prostaglandin but in the colon prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ stimulate cholinergic nerves, whereas prostaglandins E_1 and E_2 stimulate non-cholinergic excitatory nerves (Bennett, Eley & Scholes, 1968a; Harry, 1968; Bennett, Eley & Stockley, 1975a). In circular muscle of the intestine prostaglandins E and F seem to act directly on the muscle but whereas the F prostaglandins cause contraction, the E prostaglandins cause inhibition (Bennett *et al.*, 1968a; Fleshler & Bennett, 1969).

Prostaglandins may generate tone in the longitudinal muscle of guinea-pig isolated intestine (Bennett & Posner, 1971; Botting & Salzmann, 1974; Willis, Davison & Ramwell, 1974; Bennett *et al.*, 1975a) and may be necessary to maintain contractions to histamine and other substances (Eckenfels & Vane, 1972). By contrast, endogenous prostaglandin E may maintain relaxation of the circular muscle (Bennett *et al.*, 1975a). Prostaglandins E_1 or E_2 bathing the serosal surface of guinea-pig isolated ileum inhibit peristaltic activity (Bennett *et al.*, 1968b), but low concentrations (10–50 ng/ml) of prostaglandin E_1 first cause stimulation (Radmanović, 1972). Serosally applied prostaglandins E_1 or E_2 (0.3–0.5 µg/ml) in guinea-pig isolated colon, or prostaglandins $F_{1\alpha}$ or $F_{2\alpha}$ in the ileum (0.1–1 µg/ml) or colon (0.3–0.5 µg/ml) tend to stimulate circular muscle contractions when peristalsis is initiated by raising the intraluminal pressure (unpublished data). These results suggest that prostaglandins may be involved in peristalsis. Furthermore, prostaglandin E may modulate the response to cholinergic nerve stimulation (Ehrenpreis, Greenberg & Belman, 1973; Chong & Downing, 1974; Kadlec, Mašek & Šeferna, 1974; Bennett *et al.*, 1975b). We therefore studied the effects of drugs which inhibit prostaglandin synthesis (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971) on peristaltic activity in guinea-pig isolated ileum and colon. Since peristaltic activity is complex, we first evaluated various methods for quantitative assessment.

Methods

The ileum and colon were removed from freshly killed guinea-pigs, and 5–8 cm segments were cut from the mid-ileum or the colon approximately 10 cm from the anus. Peristalsis was studied by the method of Bennett *et al.* (1968a) with the following slight modifications: (1) the Marriotte bottle controlling the intraluminal pressure (zero pressure at rest to 1–5 cm water pressure) was raised at a constant rate by a linear motor for 1.5 min every 8 min (15 s to raise 4 cm, 15 s to lower); (2) in some experiments the Krebs solution expelled during peristalsis was collected in a vertical tube 25 cm long and 8 mm internal diameter, with a side-arm at the bottom to measure the hydrostatic head of pressure with a transducer. The waste outlet at the bottom of the collecting tube was closed by a relay before raising the intraluminal pressure and was opened after returning to zero pressure. This method allowed simultaneous recordings of the tone of the longitudinal muscle and the circular muscle (pressures were measured intraluminally by one or two fine polyethylene tubes) and of the propulsion of fluid. Drugs were either added to the Krebs solution bathing the serosol surface, or injected intraluminally over approximately 20 s in 1 ml 0.9% w/v NaCl (saline), usually 4 to 5 min before raising the pressure. The final concentration of intraluminally injected drugs is not known, and only the amounts injected have been stated. Control injections of saline or Krebs solution into the fluid bathing the serosol surface usually had little effect on the tissue. Control intraluminal injections sometimes variably affected peristaltic activity, so reducing the statistical significance of minor changes due to drugs.

The Krebs solution used had the following composition (g/l): NaCl 7.1, $CaCl_2.6H_20$ 0.55, KCl 0.35, KH_2PO_4 0.16, $MgSO_4.7H_2O$ 0.29, $NaHCO_3$ 2.1 and dextrose 1.0.

Tests for statistical significance

Responses to test substances were compared with controls and analysed by the median test, unless otherwise stated. All probability values refer to two-tailed tests.

Drugs

The following drugs were used: 1-acetyl-2-(8-chloro-10,11-dihydrodibenz (b,f)(1,4) oxazepine-10-carbonyl) hydrazine (SC-19220), acetylcholine perchlorate (ACh), acetylsalicyclic acid, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate (5-HT), (–)-hyoscine hydrobromide, indomethacin, nicotine hydrogen tartrate, polyphloretin phosphate (PPP), potassium chloride, prostaglandins E_1, E_2 and $F_{2\alpha}$ tromethamine salt. All concentrations of salts are expressed as base or free acid. The prostaglandins were dissolved in ethanol (0.1 ml per mg) and diluted with 0.9 ml sodium carbonate solution 0.2 mg/ml. Indomethacin 1 mg/ml and aspirin 10 mg/ml were added to saline and dissolved by adding solid sodium carbonate to pH7.

Results

Evaluation of methods to quantitate peristaltic activity

During peristaltic activity there were phasic contractions of the longitudinal and circular layers and propulsion of fluid. At least two consistent responses were obtained before a drug was added; its effect was determined by comparison with these controls and sometimes with the response after washing out the drug. Although it was clear from the trace when a drug had an effect, it was not always obvious how to express each change quantitatively. For example, reduced peristaltic activity might be accompanied by fewer but bigger circular muscle contractions, or *vice-versa.* Exact measurements of all the changes, including for example the number and height of spikes, apart from being tedious would sometimes have been difficult to evaluate (Figure 1). For example, how should sustained circular muscle contraction with no superimposed phasic activity be interpreted? Precise measurement of three traces showed reasonable correlation between separate assessments of peristaltic activity, based on the number of phasic muscle contractions, the summed heights of contraction, the mean height of contraction, the total length of pen travel in each response, and propulsion of fluid; the trends with each measurement were always in the same direction although the percentage change depended on the method of measurement and was not always consistent. In the subsequent studies we therefore measured the amount of fluid propelled and estimated changes in muscle contractility from approximate measurements of the traces.

The selectivity of drugs for studying peristalsis

Prostaglandin synthetase inhibitors. Aspirin (100–200 μg/ml) had no significant effect on submaximal contractions caused by ACh 4–10 ng/ml, histamine 10 ng/ml, nicotine, 1–20 μg/ml, or prostaglandin E_2 25–300 ng/ml ($n=6$, 1, 6 and 9 respectively). In 6 other experiments, aspirin (10–100 μg/ml) had no significant effect on responses to electrical field stimulation at 0.1 to 64 Hz. Indomethacin 1–4 μg/ml reduced contractions induced by

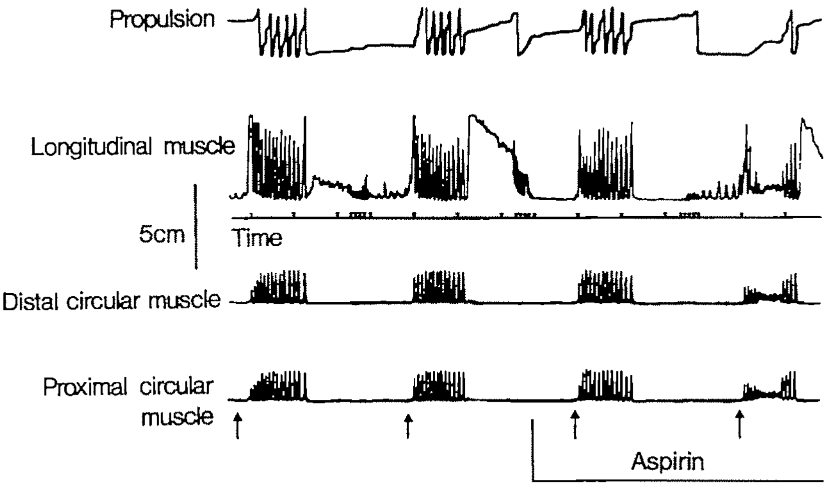

Figure 1 Peristalsis in a segment of guinea-pig isolated ileum induced by raising the intraluminal pressure from zero to 4 cm water pressure (↑). Aspirin 100 µg/ml bathing the serosal surface greatly reduced the responses in the second cycle after its administration. (In the middle of the last response there were many small contractions that are not clearly differentiated in the record). Measurement of propulsion in this experiment was by the original method of Bennett *et al.* (1968a) in which each peak represents 1 ml. Time trace, min.

prostaglandin E_2 ($n=12$) or nicotine ($n=18$) but had no significant effect on responses to ACh. With indomethacin at 1 µg/ml the reduction of the responses to nicotine was 10% (7 to 14) and of that to prostaglandin E_2 36% (32 to 44) (medians and semiquartile ranges). With indomethacin 4 µg/ml the reductions were 10% (7 to 16) for nicotine and 50% (30 to 57) for prostaglandin E_2. By comparison with saline controls the inhibitory effect was statistically significant ($P<0.03$ in every case; $n=4$ to 7; Mann Whitney U test).

Prostaglandin antagonists. Polyphloretin phosphate (PPP) 40–100 µg/ml reduced contractions of the longitudinal muscle of guinea-pig ileum induced by nicotine (1–20 µg/ml) or prostaglandin E_2 (2–200 ng/ml) but had little effect on responses to ACh (10–100 ng/ml). The highest concentration of PPP (100 µg/ml) reduced submaximal responses to prostaglandin E_2, nicotine, ACh and responses to transmural electrical stimulation (1 ms, 0.5–4.0 Hz, 3.4 V/cm for 20 s) by 90% (69 to 100) $n=7$, 70% (54 to 86) $n=6$, 5% (0 to 20) $n=8$ and 13 to 64% $n=3$ respectively. SC-19220 (4–10 µg/ml) was also non-selective: 5 µg/ml reduced submaximal contractions of the longitudinal muscle of guinea-pig ileum to prostaglandin E_2, nicotine, ACh and transmural electrical stimulation by 90% (84 to 93) $n=6$, 72% (45 to 84) $n=7$, 12% (4 to 43) $n=7$ and 19 to 54% $n=4$, respectively. Because of the depression of nerve-mediated responses, these drugs were not used to study prostaglandins in peristalsis.

Effect of prostaglandin synthesis inhibitors on peristalsis

Guinea-pig isolated ileum. Longitudinal muscle tone is slight or absent in guinea-pig isolated ileum and was slightly reduced by aspirin in only 8 out of 21 trials. Aspirin (20 to 100 µg/ml) applied serosally usually depressed peristaltic contractions of the circular and longitudinal muscles and reduced propulsion (Figure 1, Table 1). However, in 4 out of the 21 studies, the inhibition by aspirin lasted only one 8 min cycle (see Methods section) even when aspirin was still present, and even when in one experiment its concentration was increased to 1 mg/ml. The drug was usually ineffective when given after prostaglandin E_2 (0.1–0.5 µg/ml) had been added to the bath and then washed out (5 out of 6 trials in 5 experiments), and a long-lasting inhibition caused by aspirin was antagonized by adding prostaglandin E_2 (0.3–0.5 µg/ml; 4 experiments). Indomethacin (1–4 µg/ml) applied serosally acted like aspirin but inhibited peristalsis more consistently (Table 1). Longitudinal muscle tone was reduced on 2 out of 22 occasions. Unlike aspirin, indomethacin caused inhibition even when prostaglandin E_2 had previously been applied serosally and then washed out (3 experiments), but the effect of indomethacin was lost or reduced after addition of prostaglandin $F_{2\alpha}$ (0.3–0.5 µg/ml; 3 experiments), ACh (0.3–1 µg/ml; 3 experiments), or histamine (0.1 µg/ml, 1 experiment).

When injected intraluminally in a volume of 1 ml aspirin (100 µg–10 mg) had no significant effect on

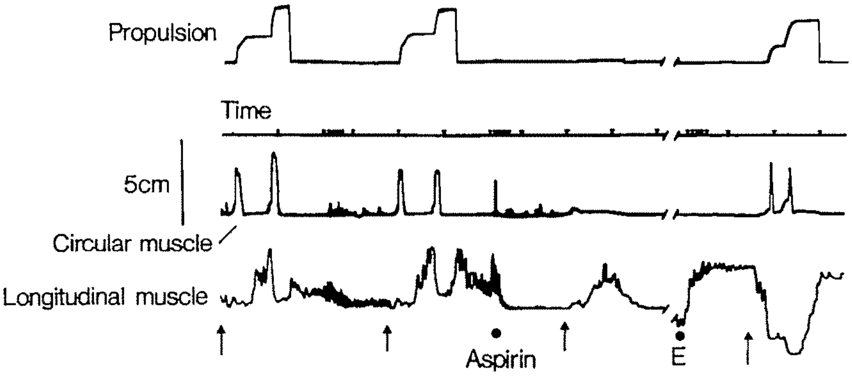

Figure 2 Peristalsis in guinea-pig isolated colon induced by raising the intraluminal pressure from zero to 5 cm water (↑). The gap in the record represents 200 minutes. During this time addition of histamine 0.2 μg/ml, 5-hydroxytryptamine 0.3 μg/ml or acetylcholine 0.2 μg/ml, after a single dose of aspirin, did not restore peristalsis, but subsequent administration of 0.3 μg/ml prostaglandin E_2 (E) antagonized the inhibition. Propulsion, 1 cm = 1 ml.

the ileum whereas indomethacin (10 or 100 μg in 1 ml) usually reduced peristaltic muscle activity and propulsion (Table 1). Longitudinal muscle tone fell with indomethacin 100 μg in 4 out of 12 experiments.

Guinea-pig colon. Aspirin 100 μg/ml bathing the serosal surface usually reduced longitudinal muscle tone and always reduced propulsion and muscle peristaltic activity. Similarly, in most preparations, indomethacin 1 or 2 μg/ml applied serosally reduced longitudinal muscle tone, muscle peristaltic activity and propulsion (Table 1).

Intraluminal administration of aspirin 50 to 100 μg in 1 ml fluid had no significant effect on peristalsis; 1 mg lowered circular muscle peristaltic activity but a tendency to reduce propulsion was not statistically significant (Table 1). Indomethacin (0.5–1 μg) administered intraluminally reduced longitudinal muscle tone in 5 out of 10 experiments and inhibited propulsion, but a tendency to reduce muscle peristaltic activity was not statistically significant.

Inhibition by serosally applied aspirin was lost or reduced after addition of prostaglandin E_2 (0.15–0.3 μg/ml; 9 experiments; Figure 2), but not by addition of ACh (0.15–0.3 μg/ml) in 5 of 6 experiments, 5-HT (0.15–0.3 μg/ml) in 3 of 4 experiments, or histamine (0.15–0.3 μg/ml) in 4 experiments. The inhibition by indomethacin was removed by addition of prostaglandin E_1 or E_2 (0.3 μg/ml) in 5 experiments.

Table 1 The effects of aspirin (ASA) and indomethacin (INDO) on percentage changes in propulsion and peristaltic activity in guinea-pig isolated intestine

						Muscle peristaltic activity %	
Tissue	*No. Expts.*	*Drug*	*Amount*	*Route*	*Propulsion*	*Circular*	*Longitudinal*
Ileum	17/12	ASA	100 μg/ml	Serosal	−33(0 to −52)*	−15(0 to −60)*	−40(0 to −70)*
	8/6	INDO	3 μg/ml	Serosal	−48(−26 to −55)*	−35(−16 to −50)*	−25(−5 to −55)*
	9/4	ASA	1 mg	Mucosal	0(0 to −12)	−10(0 to −16)	0(0 to −15)
	12/6	INDO	100 μg	Mucosal	−23(−8 to −53)†	−30(0 to −75)†	−30(0 to −60)†
Colon	14/10	ASA	100 μg/ml	Serosal	−100(−99 to −100)*	−99(−67 to −100)*	−95(−30 to −100)*
	9/8	INDO	1 μg/ml	Serosal	−100(−65 to −100)*	−90(0 to −100)*	−50(0 to −100)*
	6/4	ASA	1 mg	Mucosal	−33 (0 to −58)	−84(−74 to −90)†	−15(0 to −90)
	9/5	INDO	1 μg	Mucosal	−40 (−14 to −67)†	−72(0 to −100)	0(0 to −50)

The number of experiments represents the number of observations/number of tissues. The changes shown are medians and semiquartile ranges. Serosally applied aspirin or indomethacin significantly reduced peristaltic activity and propulsion. Londitudinal muscle tone was reduced significantly only with aspirin or indomethacin applied serosally to the colon ($P < 0.05$).

*$P < 0.01$; †$P < 0.05$ compared with saline or Krebs solution controls.

Discussion

The selectivity of drugs for the study of peristalsis

Prostaglandin synthetase inhibitors. Previous studies with guinea-pig ileum in Krebs solution (Bennett *et al.*, 1975b) indicate that indomethacin at concentrations up to 3.6 µg/ml had no significant effect on contractions of the longitudinal muscle induced by ACh, and caused at most a small depression of contractions to electrical field stimulation at 4 Hz. However, indomethacin 20 µg/ml (Chong & Downing, 1974) or 40 µg/ml (Bennett *et al.*, 1975b) caused non-selective depression. The present experiments indicate a small inhibitory effect of indomethacin 1–4 µg/ml on responses to nicotine, a greater inhibition of responses to prostaglandin E_2, but no effect on those to ACh. Aspirin 100–200 µg/ml did not significantly depress the responses to any of these drugs or to transmural stimulation. At the concentrations used, both drugs seem sufficiently free of unwanted effects to validate their use as tools to study the role of prostaglandins in peristalsis.

Prostaglandin antagonists. The prostaglandin antagonists PPP and SC-19220 were not suitable for evaluation of the role of prostaglandins in peristalsis, because concentrations required to antagonize prostaglandin effects also reduced nerve-mediated responses that may be essential for peristaltic activity. Consequently a non-selective neuronal effect might occur. On the other hand the drugs might perhaps reduce the modulation by prostaglandins of cholinergic nerve excitation (see Introduction) and this could be a common effect of drugs that selectively inhibit a neuronal action of prostaglandins.

Effects of prostaglandin synthesis inhibitors on peristalsis

Serosally applied aspirin or indomethacin reduced peristaltic activity in guinea-pig ileum and colon, but the effect of aspirin on guinea-pig ileum was sometimes temporary, despite its continued presence. Addition of prostaglandin E_2 usually prevented the effect on the ileum of aspirin but not of indomethacin, possibly suggesting the involvement of a prostaglandin pathway which was more affected by indomethacin than by aspirin. Perhaps the greater effectiveness of indomethacin is related to its ability to reduce contractions of the longitudinal muscle of guinea-pig ileum to prostaglandin E_2. The involvement of prostaglandins in peristalsis is again suggested by their ability to reverse established inhibition caused by aspirin or indomethacin. Although acetylcholine, 5-HT or histamine could also produce reversal in the ileum, they were rarely effective in the colon.

The role of prostaglandins in peristaltic activity

The above data are consistent with a role for prostaglandins in peristalsis. It is not clear whether prostaglandin E_2 and/or $F_{2\alpha}$ are important; both are thought to be present (Ambache *et al.*, 1966). They might act in various ways (as indicated in the Introduction) by modulation of intrinsic nerve activity or responses to intrinsic substances, or by maintenance of muscle tone. The inhibitory effect of added prostaglandin E_1 or E_2 in guinea-pig isolated ileum presumably represents an overwhelming action directly on the circular muscle (Bennett *et al.*, 1968b); a lower concentration of prostaglandin E_1 initially causes stimulation (Radmanović, 1972).

That some peristaltic activity occurs in the presence of aspirin or indomethacin suggests that prostaglandins are unlikely to be essential. However, it is not known if small amounts of prostaglandin synthesis occurring in the presence of the inhibitors is sufficient to allow peristalsis to occur and if peristalsis would cease if all prostaglandin synthesis or action were stopped. Nor is it known if the drugs penetrate to all parts of the tissue or are able to inhibit all biosynthetic pathways for prostaglandins. Poor penetration might explain the tendency for the synthetase inhibitors to be less active intraluminally and might account for the 30–90 min contact time required for maximal effects in isolated strips (Bennett *et al.*, 1975a). The sensitivities of prostaglandin synthesizing enzymes to indomethacin in different rabbit tissues differ over a range of 1100-fold (Bhattacherjee & Eakins, 1974). If enzymes in the gut show a spectrum of sensitivities, drugs might not inhibit prostaglandin synthesis at all sites.

Although it seems unlikely that prostaglandins are essential for peristaltic activity in guinea-pig isolated intestine, it nevertheless seems that they are needed for full activity in the colon, and possibily in the ileum. As in other sites, prostaglandins might play a modulatory role.

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DEPRESSION BY MORPHINE OF EXCITATORY JUNCTION POTENTIALS IN THE VAS DEFERENS OF THE MOUSE

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1 Intracellular recordings were made from smooth muscle cells of the mouse vas deferens. Excitatory junction potentials (e.j.ps) were evoked by stimulation of the intramural nerves.

2 Normorphine (50 nM – 5 μM) depressed the amplitude of the e.j.p. The ED_{50} was 430 nM. The latency of the e.j.p. and the resting membrane potential of the smooth muscle cells were unaffected by normorphine.

3 The depression of the e.j.p. by narcotic analgesic drugs was stereospecific.

4 Naloxone (100 nM) completely reversed the depression of the e.j.p. produced by normorphine (1 μM). Naloxone (100 nM) alone did not alter the amplitude of the e.j.p.

5 Normorphine (1 μM) did not prevent the depolarization of the smooth muscle cells produced by exogenous noradrenaline (10 μM).

6 It is concluded that narcotic analgesic drugs act directly upon the transmitter release sites to reduce the amount of noradrenaline liberated by each nerve impulse.

Introduction

Narcotic agonists inhibit the nerve-mediated contraction of the mouse vas deferens and the cat nictitating membrane. This inhibition is stereospecific and reversed by naloxone (Henderson, Hughes & Kosterlitz, 1972, 1975; Hughes, Kosterlitz & Leslie, 1975). The site of action of the narcotic agonists is thought to be prejunctional because the drugs do not reduce the contraction of the tissues to exogenous noradrenaline (Cairnie, Kosterlitz & Taylor, 1961; Henderson, 1974) but, at both sites, they greatly reduce the output of noradrenaline from the tissue which follows repetitive stimulation of its nerve supply (Henderson *et al.*, 1972; 1975; Hughes *et al.*, 1975).

Excitatory junction potentials (e.j.ps) in response to nerve stimulation have previously been recorded from individual smooth muscle cells of the mouse vas deferens (Holman, 1967; Furness & Burnstock, 1969; Furness, 1970; Bennett, 1973a). Changes in the amplitude of the e.j.p. afford a sensitive method of studying changes in the amount of transmitter released from the presynaptic nerve terminals in response to a single stimulus. The aim of the present experiments was to use this technique to examine the site of action of morphine at this autonomic neuro-effector junction.

Preliminary results of this work have been published (North & Henderson, 1975).

Methods

Male mice (TO strain), weighing 25–35 g, were killed by cervical dislocation. The vasa deferentia were dissected out and one was placed in a shallow organ bath similar to that described by Nishi & North (1973). The preparation was pinned to the floor of the bath and a portion (1–2 mm) of the outer sheath was gently removed. Glass micro-electrodes filled with 2 M KCl (tip resistance 45–90 MΩ) were used for intracellular recordings. The recorded signals were amplified, displayed and photographed in the conventional manner. The intramural nerves were stimulated by two platinum ring electrodes placed around the vas deferens approximately 2 mm apart and 5 mm from the site of recording. For studies of drug action, the preparation was stimulated with a single rectangular pulse (100 μs to 1 ms duration) at intervals of 30 seconds. The stimulus strength was adjusted so as to evoke control e.j.ps between 15 and 25 mV in amplitude. In measuring e.j.p. amplitudes, no correction was made for non-linear summation (Martin, 1955).

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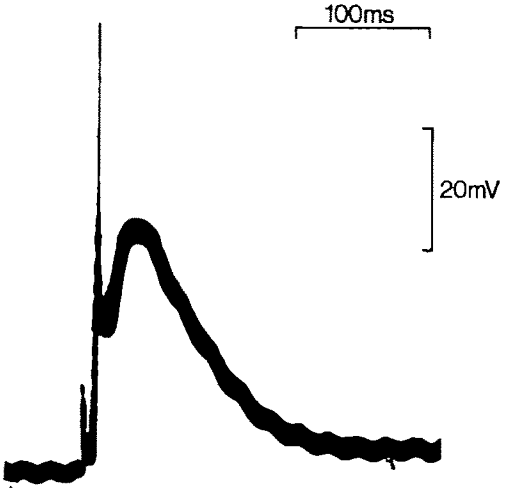

Figure 1 Evoked excitatory junction potential (e.j.p.) giving rise to an action potential. Calibrations: horizontal 100 ms, vertical 20 mV.

The preparation was perfused with a Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.19, KH_2PO_4 0.93, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2 and 5% CO_2. In those experiments in which noradrenaline was added, the Krebs solution contained ascorbic acid (100 μM) and disodium edetate (27 μM). The flow rate of the Krebs solution was 2 ml/min and the bath temperature was 37°C. Drugs used were: bretylium tosylate (Burroughs Wellcome), amphetamine sulphate (Smith, Kline & French), dextrorphan tartrate (Roche Products), hexamethonium bromide (May and Baker), hyoscine hydrobromide (B.P.), levorphanol tartrate (Roche Products), morphine hydrochloride (B.P.), naloxone hydrochloride (Endo Laboratories), (–)-noradrenaline bitartrate (BDH) and normorphine hydrochloride (Dr E.L. May). The concentrations are given in nM or μM.

Results

Characteristics of excitatory junction potentials

The smooth muscle of the mouse vas deferens is not spontaneously active and intracellular recordings were obtained from single cells for periods of up to 4 hours. The resting membrane potential of single cells was between –55 and –65 mV; the value recorded at the moment of impalement remained unchanged throughout the long impalements. Spontaneous e.j.ps were observed in most of the cells impaled. A single stimulus applied to the intramural nerves elicited an e.j.p. in every cell which was satisfactorily impaled. The latency was 6–10 milliseconds. The e.j.p. increased in amplitude as the strength of the stimulus was increased; this increase in amplitude was not smoothly graded but occurred in discrete increments, suggesting that excitation of a limited number of nerve fibres was responsible for the recorded e.j.p. With constant stimulation parameters, the e.j.ps recorded successively from adjacent muscle cells were almost identical. In most cells, the e.j.p. gave rise to a graded action potential. The threshold depolarization for action potential initiation varied between 30 and 40 mV (Figure 1).

When the intramural nerves were stimulated at 0.033 Hz there was neither facilitation nor decline of successive e.j.ps. At higher frequencies of stimulation a progressive increase in amplitude of the e.j.p. occurred during the first 5 to 10 stimuli.

The e.j.ps were unaffected by exposure of the tissue for 15 min to hyoscine (1 μM) or to hexamethonium (100 μM) although they were rapidly abolished by bretylium (50 μM). The action of bretylium was not reversed by washing out the drug, but was reversed by the addition of amphetamine (50 μM).

Effect of normorphine on the e.j.p.

The resting membrane potential of the smooth muscle cells was not changed by the addition of normorphine (50 nM–5 μM) to the perfusing solution. The apparent input resistance of the cells was determined by observing the steady state potential displacement caused by passing pulses of a known current through the recording micro-electrode by means of a bridge circuit; this was not affected by normorphine.

Normorphine reduced the amplitude of the evoked e.j.p. in a dose-dependent manner (Figure 2); it did not affect the latency of the response. Figure 2 represents the pooled data from a number of cells. The threshold concentration at which depression of the e.j.p. was observed was 50 nM and the ED_{50} (calculated from Figure 2) was 430 nM. The e.j.p. was completely abolished by concentrations of normorphine greater than 1 μM. The e.j.p. was smoothly depressed with respect to time, the depression being maximal between 3 and 6 min after changing to a solution containing normorphine (Figure 3). Upon washing out the normorphine, the e.j.p. regained its initial amplitude within a similar period of time. Depression of the e.j.p. was observed in all 37 cells to which normorphine was applied. As has previously been demonstrated on the contractions of the mouse vas deferens to nerve stimulation (Hughes *et al.*, 1975; Kosterlitz, Leslie & Waterfield, 1975) morphine and normorphine were equipotent in depressing the e.j.p.; the time course of the action of morphine was approximately twice as long as that of normorphine and for this reason normorphine was used in the majority of the experiments.

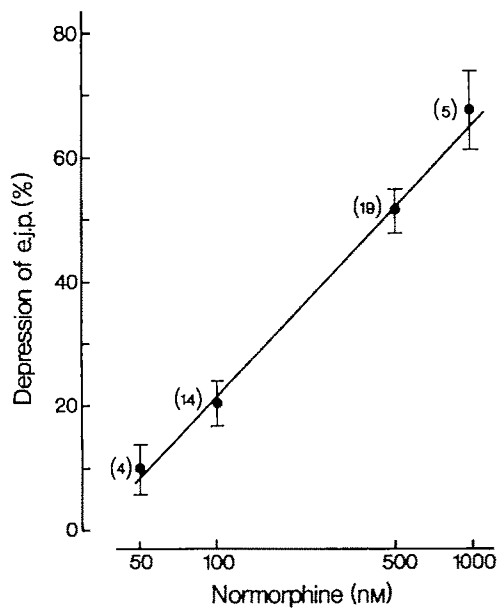

Figure 2 Dose-response curve for the depression of excitatory junction potential (e.j.p.) by normorphine. The tissues were stimulated at 0.033 Hz. Each point is the mean of the number of experiments shown in parentheses. Vertical bars represent s.e. mean. Ordinates: amplitude of the e.j.p. when maximally depressed by normorphine, expressed as a percentage of the control amplitude in the same cell. Abscissae: normorphine concentration (nM).

Specificity of narcotic agonist action

Naloxone (100 nM) was added to the bathing solution for periods of up to 20 min without any measurable change in the resting membrane potential or e.j.p. amplitude (Figure 4). However, the reduction of the e.j.p. amplitude produced by normorphine (1 μM) was rapidly reversed by changing to a solution containing naloxone (100 nM) in addition to normorphine (1 μM) (Figure 3). Levorphanol (100 nM) markedly depressed the e.j.p. amplitude whereas a ten-fold higher concentration of its (+)-isomer dextrorphan was without significant effect (Figure 5). Naloxone (50 nM) rapidly and completely reversed the depression of the e.j.p. produced by levorphanol (100 nM) (Figure 5). As on the contractions of the mouse vas deferens (Hughes *et al.*, 1975; Kosterlitz *et al.*, 1975) levorphanol was approximately twice as potent as normorphine in depressing the e.j.p. although the onset and decline of its action were much slower. Maximal depression of the e.j.p. occurred 5–10 min after changing to a solution containing levorphanol. The highest concentration of dextrorphan which was employed (5 μM) depressed the e.j.p. amplitude by not more than 15% of its control value.

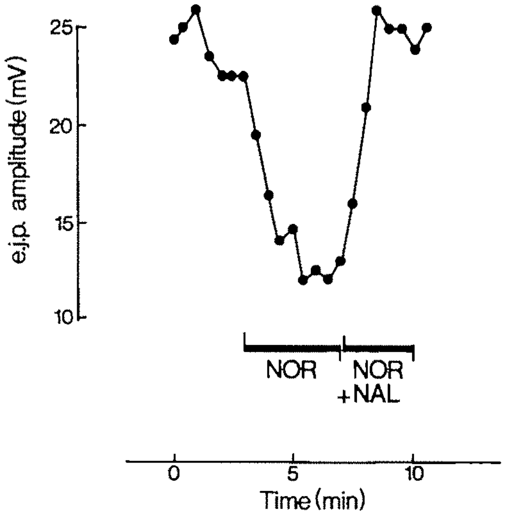

Figure 3 Reversal by naloxone of the reduction in excitatory junction potential (e.j.p.) amplitude caused by normorphine. During the periods indicated, the tissue was exposed to normorphine (NOR, 1 μM) and then to normorphine (1 μM) plus naloxone (100 nM) (NOR + NAL). Ordinates: e.j.p. amplitude (mV). Abscissae: time after impalement (min).

Action of exogenous noradrenaline

Concentrations of noradrenaline lower than 10 μM were without effect on the membrane potential of the smooth muscle cells although concentrations of less than 1 μM are capable of causing contraction (Jones & Spriggs, 1975). A similar finding has previously been noted (Holman, 1967) and may be attributed to the inability of low concentrations of noradrenaline to penetrate to the post-junctional receptor sites on the muscle cells lying deep within the tissue from which we recorded. Higher concentrations of noradrenaline often caused a violent contraction of the vas deferens which dislodged the micro-electrode. In experiments in which the micro-electrode was not dislodged, noradrenaline (10 μM) was found to cause a marked depolarization of the muscle cell membrane and a concomitant reduction in amplitude of the e.j.p. (Figure 6). The depolarization produced by noradrenaline varied markedly from cell to cell and at different times within the same cell; this may be due to slight tissue movements. However, the depolarization caused by noradrenaline was not prevented by prior exposure of the tissue to normorphine (1 μM) (Figure 6).

Discussion

The characteristics of the e.j.ps recorded in this investigation were similar to those described by Holman

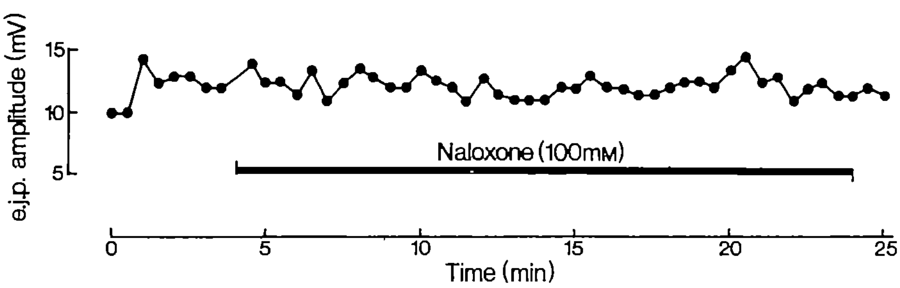

Figure 4 Absence of effect of naloxone on the amplitude of the excitatory junction potential (e.j.p.). The tissue was exposed to naloxone (100 nM) during the period indicated. Ordinates: e.j.p. amplitude (mV). Abscissae: time after impalement (min).

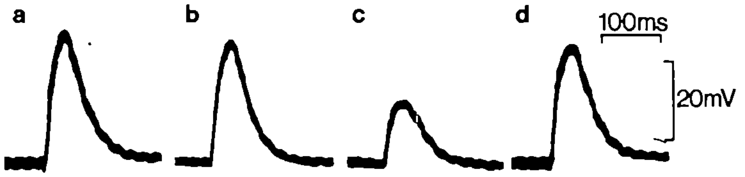

Figure 5 Effects of dextrorphan and levorphanol on the excitatory junction potential (e.j.p.). The tissue was stimulated at 0,033 Hz. (a) Control e.j.p.; (b) e.j.p. when maximally depressed by dextrorphan (1 μM); (c) e.j.p. when maximally depressed by levorphanol (100 nM); (d) e.j.p. 10 min after replacing levorphanol (100 nM) by levorphanol (100 nM) plus naloxone (50 nM). Calibrations: horizontal 100 ms, vertical 20 mV.

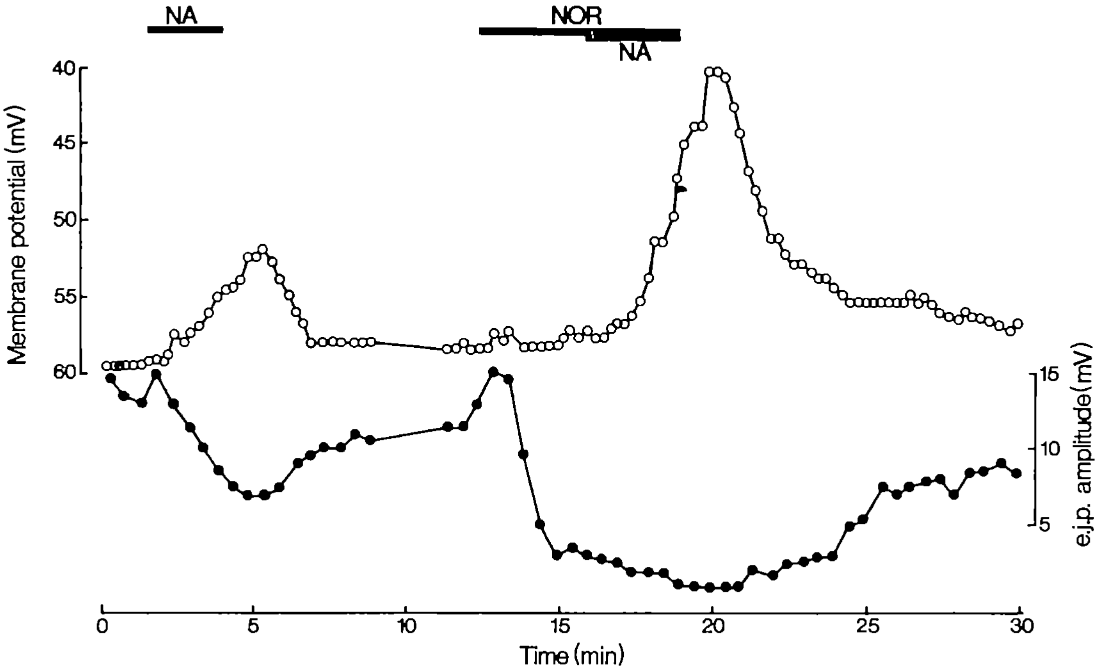

Figure 6 Effect of noradrenaline and normorphine on the membrane potential in a single smooth muscle cell. Ordinates: membrane potential (○); excitatory junction potential (e.j.p.) amplitude (●). Abscissae: time after impalement. The tissue was exposed to noradrenaline (NA, 10 μM) and to normorphine (NOR, 1 μM) during the periods indicated. The depolarization produced by noradrenaline varied markedly from cell to cell and at different times within the same cell; this may be due to slight tissue movements. This depolarization was not prevented by a concentration of normorphine which markedly depressed the e.j.p.

(1967) and Furness (1970). There is good evidence that the e.j.ps recorded from the smooth muscle cells of the mouse vas deferens are due to the release of noradrenaline from adrenergic nerve varicosities. The mouse vas deferens receives a dense adrenergic innervation (Sjöstrand, 1965). As previously described by Holman (1967), exogenous noradrenaline depolarized the smooth muscle cells and reduced the amplitude of the e.j.p. The reduction of e.j.p. amplitude was not a result of the decrease in resting membrane potential (Holman, 1967) but may have been due to an inhibition of neuronal noradrenaline release by exogenous noradrenaline (Starke, 1972). The e.j.p. is abolished by 6-hydroxydopamine (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970); it is markedly reduced by reserpine (Bennett & Middleton, 1975) but only by high concentrations of phenoxybenzamine (Bennett, 1973b). Two factors may contribute to the low potency of phenoxybenzamine. First, the mouse vas deferens receives an intimate innervation from close contact varicosities on nerve processes (Lane & Rhodin, 1964; Yamauchi & Burnstock, 1969; Furness & Iwayama, 1971). The concentration of noradrenaline within the synaptic cleft following nerve stimulation will be very large thus requiring a high concentration of phenoxybenzamine to inhibit transmission (Furness, 1974). Second, phenoxybenzamine may increase the concentration of noradrenaline within the synaptic cleft by inhibiting noradrenaline uptake (Avakian & Gillespie, 1968) and by preventing negative feedback on noradrenaline release by an action on presynaptic α-receptors (Starke, 1972). Further evidence of the adrenergic nature of the e.j.p. is obtained from our observations that the e.j.p. is abolished by bretylium; as at other adrenergic synapses (Day & Rand, 1963) the action of bretylium persisted after washing the tissue with drug-free Krebs solution but was reversed by amphetamine. The e.j.p. was unaffected by hyoscine and hexamethonium.

The action of narcotic agonists in depressing the evoked e.j.p. might be at one or more of four sites. First, it may occur at transmission between ganglion cells in the wall of the vas deferens. Such an action can be directly refuted because (a) ganglion cells are extremely sparse within the wall of the mouse vas deferens (Gabella, personal communication), (b) the absence of any effect of hexamethonium suggests that postganglionic elements are being stimulated and (c) the short latency of the e.j.p. described in this paper and by other workers (Furness, 1970) precludes a ganglionic synapse. Second, the narcotic agonists might act on conduction within the intramural nerves. The present observations that the e.j.p. amplitude was reduced in a smoothly graded manner and without change in its latency argue against this interpretation. Local anaesthetic effects of narcotics are non-specific and occur only at 1,000-fold higher concentrations (Ritchie & Armett, 1963; Kosterlitz & Wallis, 1964). Third, the narcotic agonists may directly depress transmitter release by an action at pre-junctional release sites. Fourth, the depression of the e.j.p. amplitude may be due to post-junctional effects of the narcotic agonists. With an intracellular electrode it is difficult to exclude changes in the electrical resistance of the smooth muscle cell membrane as a basis for the reduction in e.j.p. amplitude; in the present experiments, the apparent input resistance of the cells was not changed by normorphine. The observations that the noradrenaline-induced membrane depolarization and the noradrenaline-induced contraction of the isolated vas deferens (Henderson, 1974) are unaffected by normorphine make a post-junctional action of the narcotics even less likely.

The present experiments support the third site of action; namely that narcotic agonists act directly upon the noradrenaline release mechanism. This interpretation is lent weight by the observations that the noradrenaline output following repetitive nerve stimulation is markedly reduced by narcotic agonists irrespective of whether the noradrenaline output is determined by bioassay (Henderson *et al.*, 1972), or by fluorimetric (Henderson, 1974) or radiochemical (Hughes *et al.*, 1975) techniques. The inhibition of noradrenaline output is stereospecific, reversed by naloxone and occurs over the same concentration range as the inhibition of the e.j.p. Jenkins, Marshall & Nasmyth (1975) reported to a recent meeting of the British Pharmacological Society that using a bioassay technique they were unable to observe a depression of the noradrenaline output from the mouse vas deferens by morphine. At present, it is difficult to explain the difference between their findings and the well-documented evidence of the other authors.

The findings of a low ED_{50}, the ineffectiveness of the (+)-isomer and the reversibility of naloxone leave little doubt as to the receptor specificity of the action of the narcotic agonists upon transmitter release in the mouse vas deferens. An endogenous substrate for the morphine receptor (enkephalin) has recently been isolated from pig brain and guinea-pig ileum (Hughes, 1975; Hughes, Smith, Morgan & Fothergill, 1975). In the latter tissue, low doses of naloxone increase the output of acetylcholine evoked by nerve stimulation (Waterfield & Kosterlitz, 1975) and this action might be due to antagonism of endogenous enkephalin. The absence of effect of naloxone alone on the e.j.p. is evidence against the view that enkephalin normally plays a role in controlling transmitter release in the mouse vas deferens.

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A COMPARATIVE STUDY OF THE ACTION OF γ-AMINOBUTYRIC ACID AND PIPERAZINE ON THE LOBSTER MUSCLE FIBRE AND THE FROG SPINAL CORD

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1 The effects of γ-aminobutyric acid (GABA) and piperazine were compared on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord.

2 Both GABA and piperazine increased the membrane conductance of single lobster muscle fibres without changing the membrane potential; sigmoidal log dose-conductance curves for these agents were obtained and a similar model expressed the receptor interaction of both substances.

3 The actions of GABA and piperazine on lobster muscle were antagonized by picrotoxin and were Cl^--dependent.

4 In the frog spinal cord GABA depolarized the dorsal roots presumably by mimicking the activity of the transmitter depolarizing the primary afferents; sigmoidal log dose-response curves for GABA were obtained.

5 On the dorsal roots piperazine produced either depolarizations or biphasic responses; these were mainly indirect effects as was shown by experiments in the presence of tetrodotoxin (TTX).

6 The effects of GABA on the dorsal root (in TTX-treated cords) were antagonized by picrotoxin whereas those of piperazine were more resistant to this alkaloid. The GABA-induced responses appeared to be largely Na^+-dependent while both Na^+ and Cl^- seemed to mediate the effects of piperazine.

7 It is proposed that piperazine has GABA-agonist activity on lobster muscle but little GABA-like activity on the frog spinal cord.

Introduction

γ-Aminobutyric acid (GABA) is considered to be an inhibitory transmitter in several areas of the vertebrate central nervous system (Curtis & Johnston, 1974; Krnjević, 1974) and at many invertebrate synapses (Gerschenfeld, 1973). However, the characteristics of GABA-evoked responses differ according to the animal species or tissue being investigated (Curtis, Duggan, Felix, Johnston & McLennan, 1971; Bowery & Brown, 1974; Nistri, Constanti & Quilliam, 1974; Shank, Pong, Freeman & Graham, 1974; Straughan, 1974). At the lobster neuromuscular junction and in the frog spinal cord, GABA has an inhibitory action (Barker & Nicholl, 1973; Gerschenfeld, 1973), but its effects on the membrane potential and its ionic dependence differ in the two species.

The present study was prompted by the reports that piperazine, an anthelmintic agent, mimics the activity of the natural inhibitory transmitter (possibly GABA) at the *Ascaris* neuromuscular junction (del Castillo, de Mello & Moralles, 1964) and has a GABA-like agonist effect on crayfish muscle (Iravani, 1965a,b). When applied microiontophoretically, piperazine can depress the activity of rat cerebral neurones (Shinozaki & Konishi, 1970) although little attention has been paid to its action on the vertebrate central nervous system, even when isolated cases of neurotoxicity in humans have been described (Parsons, 1971). In order to examine further the possibility that the anthelmintic and central neuronal depressant actions of piperazine involve an interaction with GABA receptors, we carried out a quantitative comparison of the effects of GABA and piperazine on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord.

Methods

The lobster muscle fibre preparation

Lobsters (*Homarus vulgaris*) were obtained from a local supplier and kept in aerated artificial sea water at

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5°C until used. The opener muscle of the first or second walking leg was exposed and superfused at room temperature with crustacean Ringer solution of the following composition (mM): NaCl 522, KCl 12, $CaCl_2$ 21, $MgCl_2.6H_2O$ 5, Tris maleate 10; pH was adjusted to 7.6 with 0.1N NaOH.

The membrane potential at the centre and tendon end of a single superficial fibre in the central portion of the muscle was monitored continuously with two glass microelectrodes filled with 1.5 M tripotassium citrate connected to unity gain voltage followers (F.E.T.—operational amplifiers).

The membrane potential was recorded differentially with respect to the potential of the bathing fluid, monitored by a Ag/AgCl-Agar bath reference electrode. A third microelectrode filled with 0.6 M K_2SO_4 was inserted within 50 µm of the central voltage electrode and used to pass rectangular hyperpolarizing current pulses of constant amplitude (800 ms; 0.25 Hz) through the membrane. The current injection circuit was completed through a second bath electrode connected in series with a 40 MΩ resistor. This same electrode also kept the bath fluid at zero (earth) potential. Electrotonic potentials (e.t.ps) at the centre (V_0) and at the end (V_L) of the fibre were displayed on a Tektronix 502A oscilloscope and recorded on a Devices MX4 chart recorder. The output of the current injection circuit was also recorded on a separate channel.

Calculation of the membrane conductance

The membrane conductance of single muscle fibres was calculated by the method of Takeuchi & Takeuchi (1967) using the short cable equations (Weidmann, 1952; Vaughan, 1974). The general analysis has been fully described elsewhere (Takeuchi & Takeuchi, 1967; Feltz, 1971; Earl & Large, 1974). In the present method, the three microelectrodes were kept within the fibre throughout the experiment without causing any obvious damage to the cell. This allowed an average value of the 'resting' length/space constant ratio (L/λ) to be calculated from some 20 to 30 individual measurements made throughout the day using the relation

$$L/\lambda = \cosh^{-1}(V_0/V_L) \qquad (1)$$

V_0/V_L was the ratio of hyperpolarizing e.t.ps at the middle (V_0) and end (V_L) of the fibre. This average value of L/λ was then used in the calculation of the resting membrane conductance at any instant from

$$g_m L = (I_0/2V_0)(L/\lambda)\coth(L/\lambda) \qquad (2)$$

where g_m was the conductance per unit length ($mho.cm^{-1}$) and I_0/V_0 the input conductance (mho). The current/voltage relation of the lobster fibre was sufficiently linear in the hyperpolarizing direction to allow the input conductance to be estimated directly from the ratio of current to e.t.p. amplitude at the centre of the fibre without introducing serious error (imposed hyperpolarizations were always $\leqslant$ 15 mV).

The modified length/space constant ratio (L/λ^*) in the presence of GABA or piperazine was calculated by an iterative procedure (Earl & Large, 1974) using the average L/λ and the ratio of e.t.ps at the centre of the fibre in the presence (V_0^*) and absence (V_0) of drug. L/λ^* and V_0^* were then substituted into eqn (2) to give the modified conductance g_m^*L and the actual change in membrane conductance was obtained by subtracting the resting conductance value calculated immediately before the addition of the drug

$$\Delta g_m L = g_m^*L - g_m L \qquad (3)$$

The iterative method for calculating L/λ^* avoided the use of V_L^* which, during large conductance changes, often became too small to measure with accuracy.

The frog spinal cord preparation

Frogs (*Rana temporaria*) were kept in a tank with circulating water at 5°C until used. The spinal cords were removed following dorsal laminectomy, hemisected and fixed on the bottom of a 0.5 ml bath as previously described (Nistri, 1975). The frog Ringer solution had the following composition (mM): NaCl 109, KCl 4 mM, $CaCl_2$ 1.5, $NaHCO_3$ 1.27, glucose 4; and was gassed with 95% O_2 and 5% CO_2 (pH 7.2). Stimulations and recordings were made through the VIIIth or IXth pair of lumbar roots placed in paraffin-filled side chambers. The whole preparation was maintained at 13 to 14°C throughout the experiment. Rectangular pulses (1 Hz; 0.1 ms; supramaximal voltage) were delivered through platinum electrodes.

Two types of potential were recorded: (1) the ventral root potential (VRP) evoked by orthodromic stimulation of the adjacent dorsal root, and (2) the dorsal root potential (DRP) evoked by antidromic stimulation of the adjacent ventral root. These potentials were recorded differentially with Ag/AgCl-Agar electrodes, displayed on a storage oscilloscope and a 502A Tektronix oscilloscope and photographed with a Polaroid camera. The potentials and the root d.c. polarization level were recorded on a two channel pen recorder.

Drugs

All the drugs were adjusted to the pH of the Ringer solutions and applied to the two preparations via the bathing fluid. The drugs used were obtained from the following sources; GABA from B.D.H., piperazine citrate, tetrodotoxin, picrotoxin and sodium glutamate from Sigma; dibenamine hydrochloride was kindly donated by Smith, Kline & French. The doses of piperazine quoted in the text on a molar basis refer to the citrate salt. Dibenamine hydrochloride was diluted

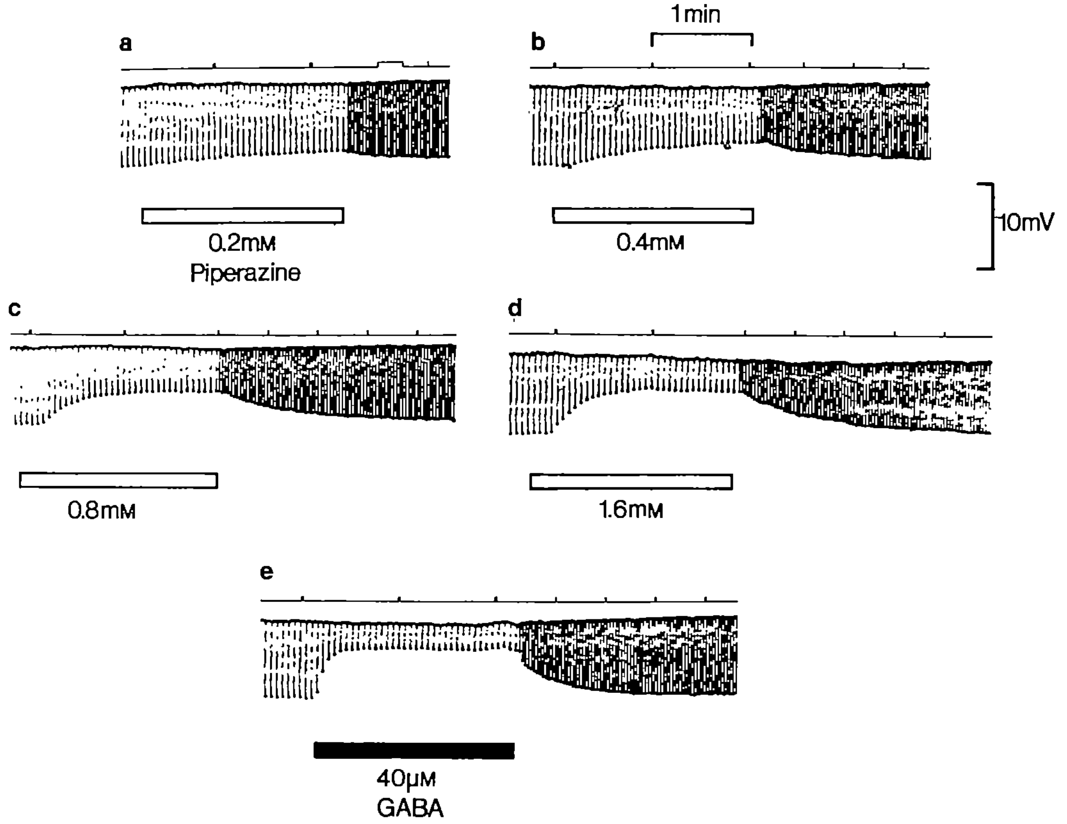

Figure 1 The effect of piperazine (0.2 mM to 1.6 mM, open bars) on the hyperpolarizing electrotonic potentials (downward deflections) recorded at the centre of a single lobster muscle fibre (resting potential = −76 mV) in response to intracellular current pulses applied via a second central microelectrode (800 ms; 1.45×10^{-7}A). (a to d) reduction in amplitude of the hyperpolarizing potentials (indicating increases in membrane conductance) during application of successively increasing concentrations of piperazine (with intermediate washing). In (e), the muscle was bathed with γ-aminobutyric acid (GABA) solution (40 μM, filled bar) over a similar period. Note that the onset/decline rates for the action of piperazine were much slower than those for GABA. Chart speed was halved during decline of responses.

immediately before use from freshly made stock solutions (10% w/v) in 90% ethanol. In this case GABA solutions contained the same small amount of 90% ethanol and produced responses no different from those obtained with control doses.

Results

Effects of GABA or piperazine on lobster muscle fibres

The bath application of GABA (5 μM to 640 μM) produced a reversible and dose-related increase in the membrane conductance with little or no change in the resting potential. Such an effect, already described in our laboratory (Constanti & Quilliam, 1974; Nistri & Constanti, 1975), had a typically fast onset and did not fade despite the continued presence of GABA (see below). A typical example of GABA action on the e.t.p. recorded from a single fibre is shown at the bottom of Figure 1. Figure 1 also shows the effects of piperazine on the same fibre. As with GABA, this compound (0.1 to 3.2 mM) produced dose-related increases in membrane conductance unaccompanied by a change in membrane potential, and with no visible desensitization. Piperazine however, was much less potent than GABA on a molar basis. Moreover, both the onset and the decline of the effects of piperazine were slower than those of GABA, even when similar conductance changes were obtained in each case. Piperazine responses usually required about 2 min to attain equilibrium and 5–10 min for recovery, whereas equilibrium GABA responses were normally attained within 30 s of changing from control to test solution and required 4 to 5 min for recovery of the resting conductance.

Owing to the very small size of the e.t.p. during large conductance changes, the maximal conductance could not be measured with accuracy. A normalization procedure was therefore adopted whereby all GABA- and piperazine-induced conductance changes measured on any single fibre were expressed as fractions of the conductance change produced by 40 μM GABA in that fibre. The normalized conductance increase expressed as ΔG_N was then used to construct normalized log dose-conductance curves. This procedure also allowed the pooling of conductance data from different muscle fibres, of varying diameter, length and resting conductance.

Figure 2a shows the normalized log dose-conductance curves for GABA and piperazine, the latter curve having been displaced one log unit to the left along the abscissa scale to facilitate comparison. Each point represents the mean (with standard error) of 5 experiments in which GABA and piperazine curves were constructed on a single fibre. In the range studied, the piperazine curve appeared to be parallel with the GABA curve suggesting a similar mechanism of action at receptor level. When the dose-conductance curves for both compounds were plotted on linear coordinates (not shown), the curves showed an initial sigmoidicity, suggesting that more than one molecule of each drug was involved in the receptor interaction; in addition the Lineweaver–Burk double-reciprocal plots of these data showed a marked non-linearity.

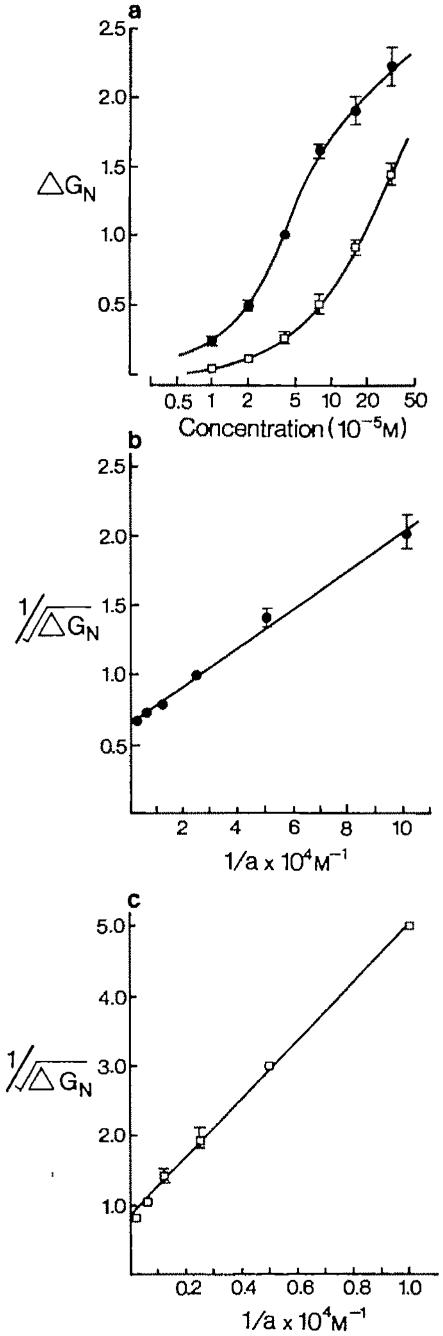

Figure 2 (a) Normalized log dose-conductance curves for γ-aminobutyric acid (GABA) (●) and piperazine (□). Points represent mean of 5 experiments. Vertical lines show s.e. mean. Errors not shown were within the size of the points. Ordinate scale represents normalized increase in membrane conductance (ΔG_N); abscissa scale gives concentration of drug added to the superfusing solution. ΔG_N was calculated with respect to the 40 μM (4×10^{-5}M) GABA response (see text). Note that the piperazine curve has been displaced one log unit to the left along the abscissa scale in order to facilitate comparison. (b and c); Double reciprocal transformations ($1/\sqrt{\Delta G_N}$ vs $1/a$) of the normalized GABA and piperazine conductance curves (data from Figure 2a). The plots were drawn on the basis of the two independent binding-site models. ΔG_N represents normalized conductance increase and 'a' is the drug concentration (10 μM; 10^{-5}M). Error intervals indicated are the transformed standard errors. (●) GABA plot: concentration range represented, 10 μM (10^{-5}M) to 320 μM (3.2×10^{-4}M). Weighted regression line had slope $= +0.143$: intercept $= 0.639$: $r = 0.998$: Estimated value of $K_{GABA} = 22.4$ μM (2.24×10^{-5}M). (□) Piperazine plot: concentration range 0.1 mM (10^{-4}M) to 3.2 mM (3.2×10^{-3}M) (note difference in ordinate and abscissa scales). Weighted regression line had slope $= +4.238$; intercept $= 0.822$; $r = +0.999$. Estimated value $K_{Pip} = 516$ μM (5.16×10^{-4}M).

Model for GABA or piperazine receptor interaction in the lobster muscle

Takeuchi & Takeuchi (1967; 1969) proposed that at least two molecules of GABA were required for the activation of the unit conductance change on crayfish muscle and they used a high-cooperativity receptor model to describe their data. In the present case, a better overall fit to the GABA data was provided by a simple two independent binding-site model (for a discussion on different receptor models see Werman, 1969) although this does not imply that the independent model is more physically likely than any other. This model assumed the presence of two equivalent agonist binding sites at the receptor, either of which could initially interact with a GABA molecule. The binding of the first molecule was considered not to influence the binding of the second, and when both sites had been activated, the unit conductance channel would be opened. The properties of the model can be represented by the equation:

$$\Delta g/\Delta g_{\max} = [a/(a+K)]^2 \qquad (4)$$

where $\Delta g/\Delta g_{\max}$ = fractional equilibrium conductance changes (measured as $\Delta g_m L$), a is concentration and K is the apparent agonist/receptor dissociation constant (= 1/affinity constant). A convenient check for conformity with this model was provided by the double-reciprocal transformation of eqn (4). However, since all responses were normalized with respect to 40 μM GABA, it was also necessary to normalize eqn (4) thus:

$$\Delta G_N = [a/(a+K)]^2/[4/(4+K)]^2$$

Taking the square root of the reciprocal gives:

$$1/\sqrt{\Delta G_N} = [\alpha + \alpha K \cdot (1/a)] \qquad (5)$$

where $\alpha = 4/(4+K)$. A plot of $1/\sqrt{\Delta G_N}$ vs $1/a$ should thus be linear with slope (αK) and intercept α. K can then be estimated from the ratio slope/intercept.

Figure 2b and c shows such double-reciprocal transformations of normalized piperazine and GABA dose-conductance curves presented in Figure 2a. In each case, the regression lines were estimated using a weighted linear regression analysis so that equal emphasis could be provided for all the points along the line. Each point was assigned a weight = 1/var (ΔG_N). Both plots were linear in the concentration ranges studied in good agreement with the proposed model, but the piperazine (Pip) double-reciprocal plot had the steeper slope. The values of K_{GABA} and K_{Pip} estimated from the slope/intercept ratio were 22.4 μM (2.24×10^{-5} M) and 516 μM (5.16×10^{-4} M) respectively, indicating the relatively lower affinity of piperazine for the GABA receptor. If both GABA and piperazine could produce the same maximal conductance change, then the ordinate intercepts should be identical. However, the intercept value of the piperazine plot (0.82) was slightly higher than that from the GABA plot (0.64), possibly indicating that piperazine produces a smaller maximum than GABA.

Effects of low Cl^- solution on GABA and piperazine-induced responses

The absence of membrane potential changes during the action of GABA or piperazine suggested a similarity in the reversal potentials. In crustacean muscle, GABA-evoked conductance changes involve an increase in Cl^- permeability (Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1967). In order to determine whether the conductance increase produced by piperazine was also Cl^- mediated, some experiments were carried out in which the external Cl^- (586 mM) of the normal solution was reduced to 325 mM; the impermeant isethionate was used as a substitute. Approximately equieffective doses of piperazine and GABA having no effect on membrane potential in normal solution (Figure 3a and b), when applied soon after changing to low Cl^- produced almost identical membrane depolarizations (Figure 3c and d). These depolarizations were reversed to hyperpolarizations (about 2 mV) on returning to a normal solution (Figure 3e and f). These changes in membrane potential were in a direction consistent with the imposed change in the Cl^- equilibrium potential, suggesting that both GABA and piperazine were producing similar changes in Cl^- permeability.

Combination experiments on the lobster muscle

Iravani (1965a,b) reported that piperazine was a partial agonist at the crayfish GABA receptor. At low GABA concentrations, piperazine increased the GABA effect while at high GABA concentrations the effect was decreased. This phenomenon was not observed in the present case. The result of a typical GABA-piperazine combination experiment is shown in Figure 4a. The GABA curve was first measured, then it was repeated with various concentrations of GABA combined with a fixed concentration of piperazine. The combination of GABA with piperazine produced approximately additive effects with no evidence of mutual inhibition at high GABA doses. Similar results were obtained with higher fixed piperazine concentrations.

Effects of picrotoxin on the GABA and piperazine-induced responses

Picrotoxin, a potent GABA antagonist at the crustacean neuromuscular junction (Takeuchi & Takeuchi, 1969; Constanti & Quilliam, 1974; Earl & Large, 1974; Nistri *et al.*, 1974; Shank *et al.*, 1974), was also tested as an inhibitor of piperazine-induced responses. Figure 4b shows the piperazine dose-

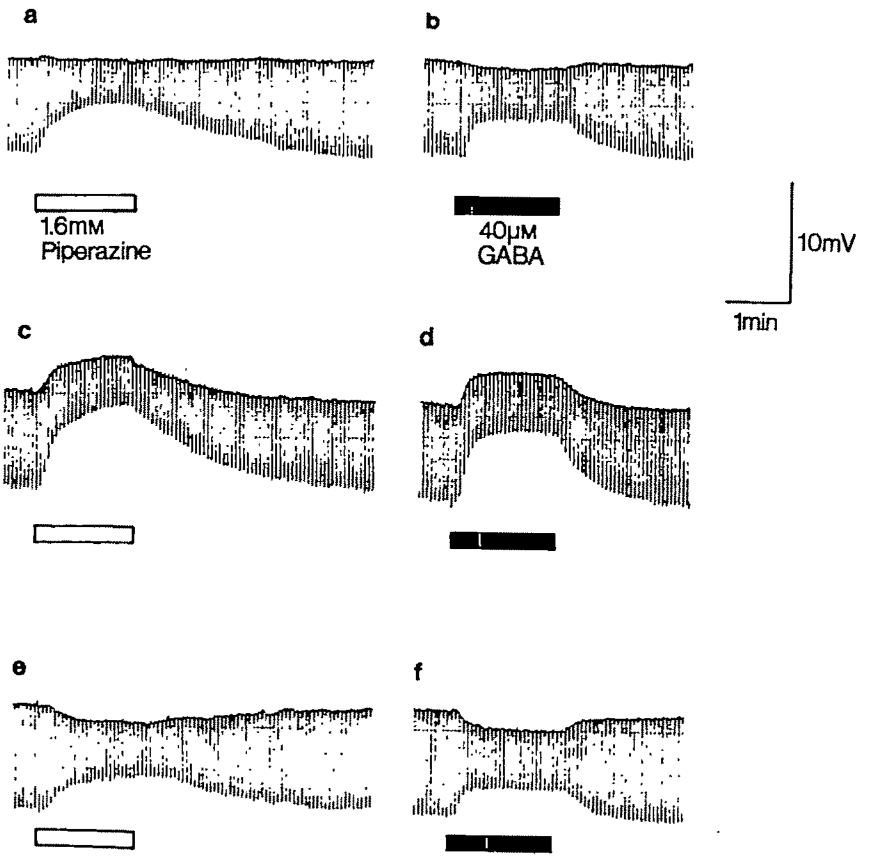

Figure 3 Hyperpolarizing electrotonic potentials (downward deflections) recorded at the centre of a single lobster fibre in response to central intracellular current pulses. (800 ms; 1.25×10^{-7}A). Records show effect of changing from normal external solution (586 mM Cl^-) to a low chloride (325 mM) solution chloride replaced with isethionate) on the membrane potential and conductance changes produced by γ-aminobutyric acid (GABA, 40 µM-filled bars) and piperazine (1.6 mM-open bars). (a,b) Control matched responses to piperazine and GABA respectively, measured in normal solution; (c,d) 5 and 10 min respectively after changing to low chloride solution, both piperazine and GABA now produced membrane depolarizations; (e,f) 5 and 10 min respectively after returning to normal bath solution, both piperazine and GABA now hyperpolarized the membrane.

conductance curve in normal solution and in the presence of picrotoxin (0.5 µM).

This concentration has been previously shown (Constanti & Quilliam, 1974) to displace the GABA curve in a non-parallel fashion with a reduction of the apparent maximal conductance change of about 65%. Figure 4b shows that the piperazine curve was also displaced to the right, although in the range studied there was no indication of a maximum.

According to Furchgott (1966) the alkylating agent dibenamine will bind irreversibly to a wide variety of membrane receptors. It was therefore of interest to test the effect of GABA in the presence of dibenamine (0.1 mM). However, despite the high concentration used, no consistent antagonism could be demonstrated.

Effects of GABA or piperazine on the frog spinal cord.

Bath application of GABA (0.1 mM) or piperazine (0.1 mM) produced a reversible depression of the VRP and DRP (Figure 5), but the onset and the decline in rates of action of piperazine were slower than those of GABA. The GABA-induced decrease in VRP was associated with either a depolarization or a hyperpolarization of the ventral root while piperazine often depolarized the same root. The decrease in DRP amplitude produced by GABA was always

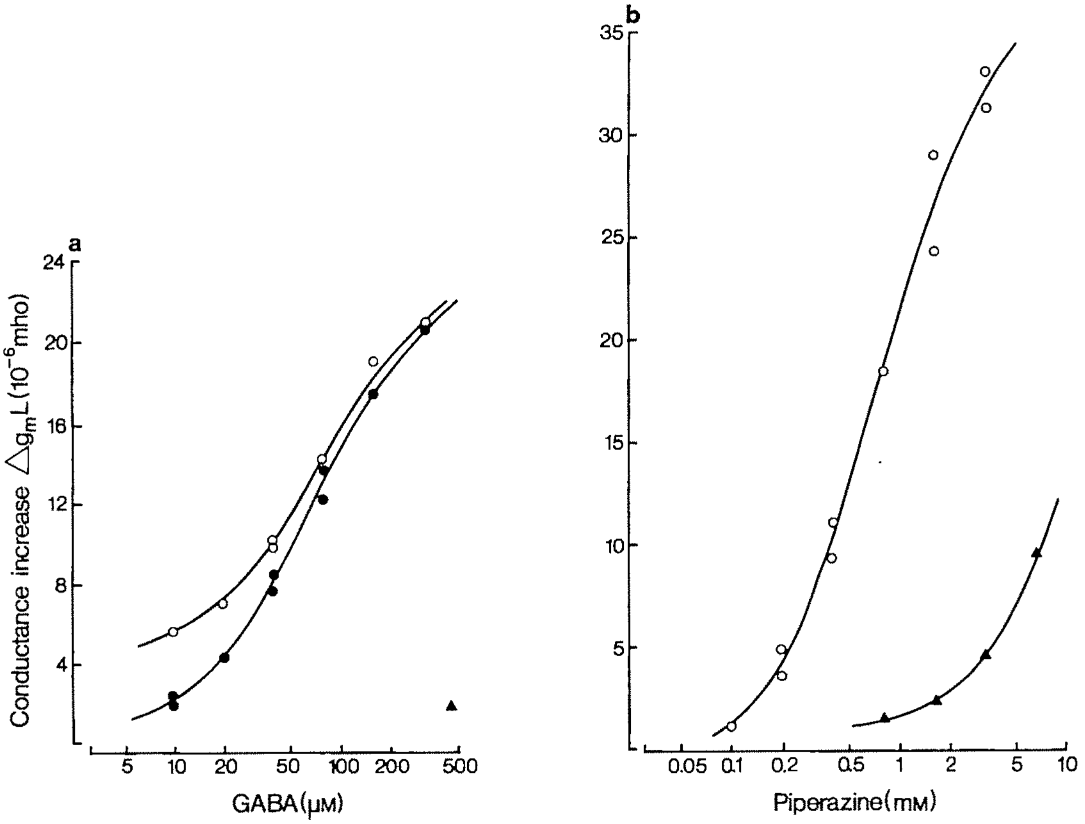

Figure 4 (a) Interaction between γ-aminobutyric acid (GABA) and piperazine. (●), Control GABA log dose-conductance curve measured in normal solution. Ordinate scale represents the increase in membrane conductance ($\Delta g_m L$) where g_m is the membrane conductance per unit length, and L is the half-length of the muscle fibre. Abscissa scale gives the concentration of applied GABA. (O), GABA 'combination' curve obtained by combining various concentrations of GABA with a fixed concentration (0.4 mM) of piperazine. (▲), Conductance change produced by 0.4 mM piperazine. All measurements were made on the same fibre. (b) Effect of picrotoxin on the piperazine log dose-conductance curve. (O), Piperazine-evoked increase in membrane conductance ($\Delta g_m L$) measured in normal solution; (▲), in 0.5 μM picrotoxin. Measurements were made on a different fibre from that of Figure 4a.

accompanied by a dorsal root depolarization while piperazine produced either a biphasic response consisting of an initial small hyperpolarization followed by a depolarization, or a pure depolarizing response. Since the action of GABA on the dorsal root was very consistent and is considered to be implicated in the depression of the primary afferents (Barker & Nicholl, 1973), the subsequent experiments dealt with the dorsal root responses to GABA. In order to avoid indirect synaptic effects from interneuronal activity the cord was treated with tetrodotoxin (TTX; 1 μg/ml) and the action of GABA was observed as a change in the d.c. polarization level of the dorsal root. Such responses therefore represented electronic spreading of the depolarization along the root (Barker & Nicoll, 1973). In the TTX-treated cord the GABA-evoked depolarizations were dose-related and had a very fast onset. However, in the continued presence of GABA, these responses declined rapidly suggesting desensitization (see inset of Figure 6a). The GABA log dose-response curve was sigmoidal (Figure 6a) but the apparent maximum was influenced by tissue desensitization. GABA dose-response curves in cords not treated with TTX had a very similar shape to those in the presence of this toxin, suggesting that any indirect effect of GABA on the dorsal root was minimal. The dorsal root responses to GABA were closely reproducible even after many hours provided that 8–10 min elapsed between each administration of this substance.

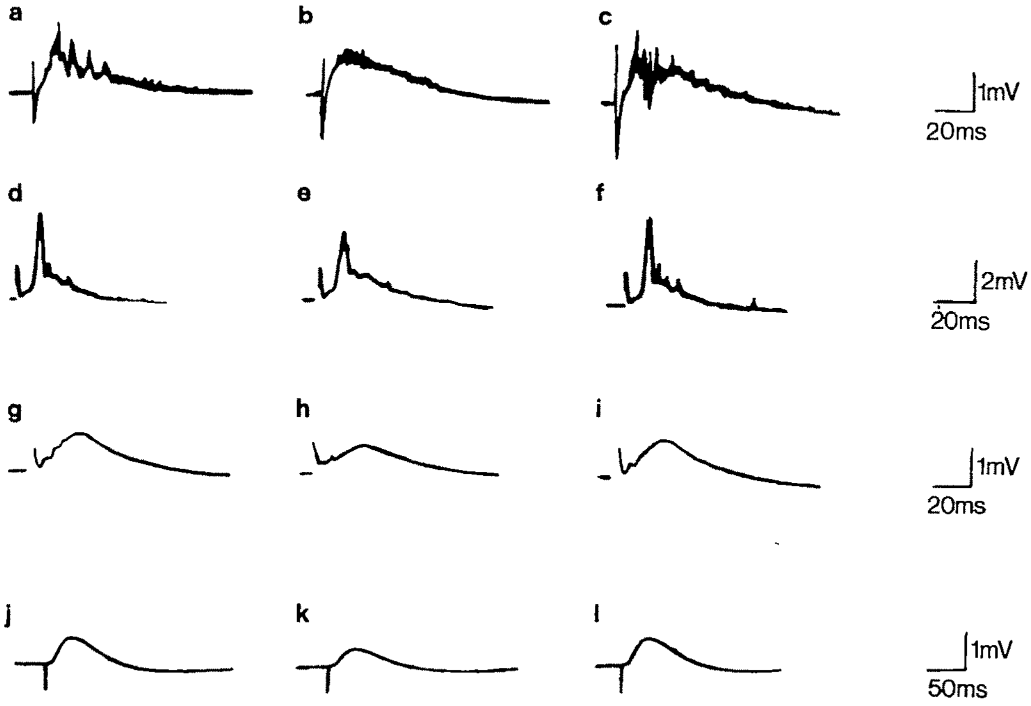

Figure 6 Effects of γ-aminobutyric acid (GABA) or piperazine on the frog ventral root potential (VRP) and dorsal root potential (DRP). (a and d) Control VRPs; (b and e) VRPs 10 min after GABA (0.1 mM) or piperazine (0.1 mM) respectively; (c and f) recovery (20 min later); (g and j) control DRPs; (h and k) DRPs after 10 min in the presence of GABA (0.1 mM) or piperazine (0.1 mM) respectively; (i and l) recovery (20 min later).

The dorsal root responses to piperazine were more variable than those to GABA. As previously mentioned, biphasic effects (hyperpolarizations followed by depolarizations) were often observed (see Figure 8). A decline of the response to piperazine during the continued application of the drug was not easily found (see inset in Figure 7). The cord was usually less sensitive to piperazine than to GABA on a molar basis and a considerable variability in the cord sensitivity to piperazine was encountered.

When piperazine log dose-response curves were compared in the presence or in the absence of TTX, a marked depression of the curve in the presence of TTX was noted (Figure 7). In order to ensure reproducibility of the dorsal root responses, the doses of piperazine had to be administered every 12–15 minutes.

In an attempt to analyze the nature of the GABA and piperazine interaction with the receptor sites in TTX-treated cords, the dose-response curves previously obtained were transformed into log-log plots. This type of analysis is useful when the tissue responses are recorded as depolarizations rather than as conductance changes (Werman, 1969). In the range studied, the GABA or piperazine log-log plots were linear with a limiting slope of 0.55 and 0.82 respectively. This suggested that one GABA or piperazine molecule was interacting with a single receptor site.

Effects of picrotoxin or ionic substitutions on the cord responses

The effect of picrotoxin (10 μM) on the GABA or piperazine action was tested in TTX-treated spinal cords. Although picrotoxin is a well-known GABA antagonist on the frog dorsal root (Barker & Nicoll, 1973), conventional log dose-response curves of this phenomenon have not been presented to show the mechanism of inhibition. Figure 6b shows that in the presence of picrotoxin the GABA dose-response curve was strongly depressed and shifted to the right whereas the piperazine curve was less affected (see Figure 7). The action of picrotoxin was slowly reversible over a period of 90 min or more.

High concentrations of dibenamine (up to 0.5 mM for 30–45 min) were used in an attempt to block the effects of GABA or piperazine. However, no clear

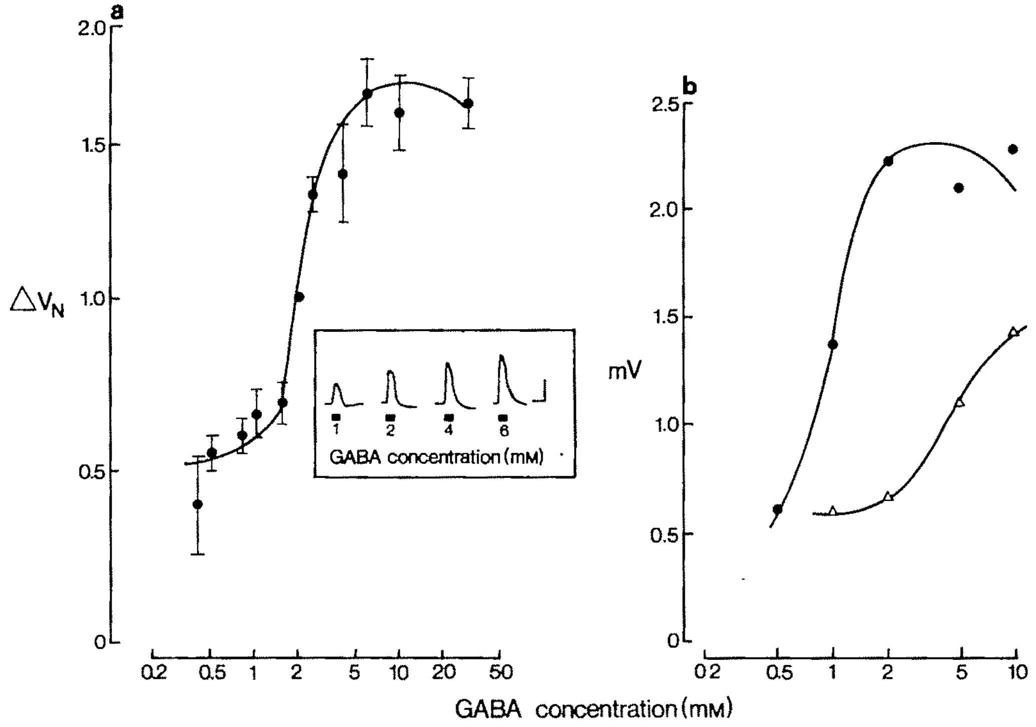

Figure 6 (a) Normalized log dose-response curve for γ-aminobutyric acid (GABA) obtained from the dorsal root of a tetrodotoxin (TTX)-treated cord. Each point (●) is the mean of 9 experiments. Vertical lines show s.e. mean. Abscissa scale: GABA concentration; ordinate scale: normalized depolarizations (ΔV_N was calculated by dividing all GABA depolarizations of any single cord by the response to 2 mM GABA in that cord). Inset: examples of GABA-induced depolarization on the dorsal root. In this and the following figures depolarizations are indicated by upward deflections of the pen. (Calibration; 1 mV; 1 min). (b) Log dose-response curves for GABA (●) and GABA in the presence of picrotoxin (△) obtained from a dorsal root of a TTX-treated cord. Picrotoxin was applied at a concentration of 10 μM for 20 minutes. Note the depression and shift of the GABA curve.

antagonism of the actions of these substances was found.

The ionic dependence of the GABA or piperazine effects on TTX-treated cords was also tested (Figure 8). Ninety per cent substitution of the Na^+ content of the bathing medium with Li^+, abolished the effects of GABA after 60 min whereas the biphasic effect of piperazine was converted into a simple hyperpolarization. No potentiation of the actions of these two substances was observed, in contrast with the report of Nishi, Minota & Karczmar (1974). The 90% substitution of external Cl^- with the impermeant isethionate ion did not alter the depolarization produced by GABA although the after-hyperpolarization was enhanced. However the biphasic response to piperazine was converted into a depolarization.

In contrast, the equimolar substitution of external Ca^{2+} with Mg^{2+} did not greatly alter the effect of GABA or piperazine. In low Na^+ medium the depolarization of the dorsal root produced by glutamate, a potent excitatory amino acid, was also abolished (not shown).

Combination experiments in the frog cord (tetrodotoxin-treated)

Following the procedure described for the lobster preparation, GABA-piperazine combination experiments were carried out to test for possible mutual hindrance. The combination of GABA with a fixed dose of piperazine (2.5 mM) produced approximately additive effects over most of the GABA concentration range. However, in view of the strong tissue desensitization to high doses of GABA, it was not possible to reach any definite conclusion about mutual interaction on this preparation.

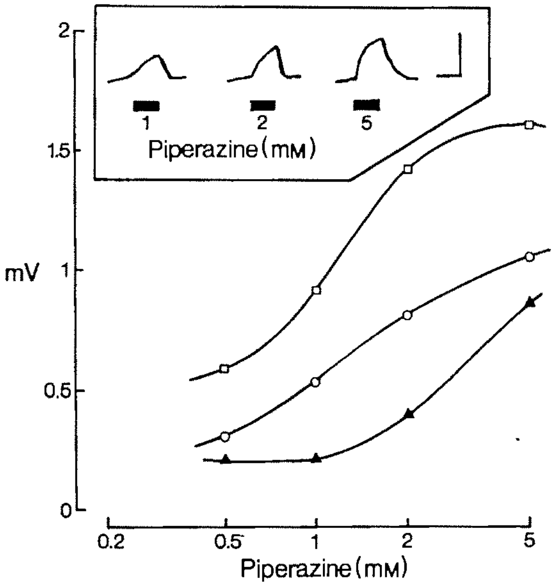

Figure 7 Log dose-response curves for piperazine obtained from a dorsal root of the frog spinal cord. Abscissa scale: piperazine concentrations; ordinate scale: depolarizations (mV). (□) indicates the curve in the absence of tetrodotoxin (TTX); (O) in the presence of 1 μg/ml TTX; (▲) in the presence of TTX and picrotoxin (10 μM for 20 minutes). Inset: examples of depolarizations produced by piperazine on a dorsal root of a TTX-treated cord. (Calibration: 1 mV; 1 min).

Discussion

Similar actions of GABA and piperazine on the lobster muscle

On the lobster muscle fibre the conductance responses to the application of GABA or piperazine had several points in common. First, both compounds produced a reversible and dose-related increase in membrane conductance without changing the membrane potential. Second, the log dose-conductance curves for the two drugs were well fitted by an independent binding model, according to which two agonist molecules were supposed to bind to a single receptor site without mutual interaction. Thirdly, an increase in Cl^- permeability appeared to mediate the effects of GABA and piperazine and fourthly, the actions of both drugs could be antagonized by picrotoxin. Piperazine thus behaved like a GABA agonist with lower affinity for the receptor than GABA itself ($K_{\mathrm{Pip}} = 516\ \mu\mathrm{M}$; $K_{\mathrm{GABA}} = 22.4\ \mu\mathrm{M}$), despite the absence of any structural similarity between the piperazine and GABA molecules. The above results are in general agreement with those of del Castillo *et al.* (1964) who suggested that the paralyzing effect of piperazine on *Ascaris* muscle was mimicking the action of the natural inhibitory transmitter (possibly GABA) although piperazine was 100 times less potent than GABA on this preparation.

According to the mass action law, when two drugs of similar efficacy (Stephenson, 1956) compete for a common receptor site, the combined effect of the two drugs would be a competitive synergism (Ariëns & Simonis, 1964). If one of the drugs were acting as a partial agonist, then mutual hindrance would occur (see also Constanti & Quilliam, 1974). The results of the present combination experiments would therefore suggest that GABA and piperazine were acting as agonists of similar efficacy although of different affinity. The only major difference between the effects of GABA and piperazine was the slow onset and offset rate of action of the latter. This might be due to a slow diffusion rate of this compound into and out of the region of the receptors. Alternatively, piperazine may be unable to share with GABA the tissue uptake process that may be important in terminating the action of this amino acid (Curtis & Johnston, 1974). Another possibility could be that piperazine dissociates from the receptors at a slower rate than GABA. All these hypotheses, which can also be proposed to explain the slow onset and offset of the responses observed in the frog spinal cord, need to be confirmed experimentally.

Differences between the actions of GABA and piperazine on the frog cord

In the frog spinal cord GABA and piperazine depressed both VRP and DRP. To clarify the mechanism of this effect, changes in dorsal root d.c. levels were also examined. Despite the presence of TTX the dorsal root responses to GABA always consisted of a depolarization with characteristic fading which suggested rapid receptor desensitization. The log dose-response curve for GABA was steep with a maximum difficult to establish with certainty. The ventral root responses were more variable consisting of hyperpolarizations or depolarizations according to the preparations. On the dorsal (and sometimes on the ventral) roots piperazine often produced biphasic responses (hyperpolarizations followed by depolarizations) with little fading. These depolarizing components of the piperazine effect gave log dose-response curves less steep than those for GABA, and the effect of piperazine was found to be largely mediated by interneurones since the addition of TTX strongly reduced it. Picrotoxin antagonized the effect of GABA in an apparently non-competitive fashion but was less effective in reducing the action of piperazine. The slopes of the log-log plots for GABA and piperazine (0.55 and 0.82 respectively) may be interpreted as one molecule of GABA or piperazine interacting with the spinal receptors.

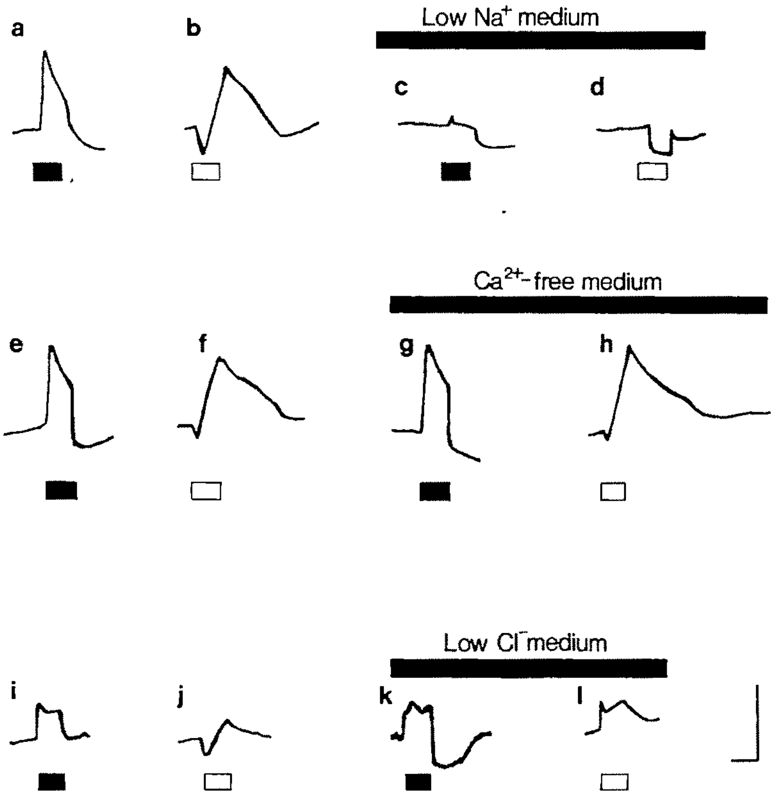

Figure 8 Effects of ionic replacement on γ-aminobutyric acid (GABA) (filled bars) or piperazine (open bars) evoked dorsal root responses of tetrodotoxin (TTX)-treated cords. (a and b) control responses to GABA (2.5 mM) and piperazine (2 mM); (c and d) responses to the same doses after 60 min in low Na$^+$ medium (90% substitution made with equimolar Li$^+$); (e and f) recovery 60 min later; (g and h) responses to the same doses after 30 min in Ca^{2+}-free medium (substitution made with equimolar Mg^{2+}); (i and j) control responses to GABA (3 mM) and piperazine (2 mM); (k and l) responses to the same doses after 30 min in low Cl$^-$ medium (90% substitution made with equimolar isethionate). Calibration bars for all responses: 1 mV; 1 min. Note the biphasic effect of piperazine in (b) and (j).

In the case of GABA at least, the low value of this slope might depend on the rapid desensitization and thus not express the actual number of binding molecules. The Na$^+$ dependence of the action of GABA on the dorsal root has already been described (Barker & Nicoll, 1973) and found also in our study. Nishi *et al.* (1974) were unable to find a Na$^+$ dependence and suggested that Cl$^-$ was the ion involved. In the present report a blockade of the effect of GABA was seen in a low Na$^+$ but not in a low Cl$^-$ medium. Therefore, although extracellular recordings cannot provide conclusive evidence about the ionic species involved in a depolarization, the effect of GABA on the dorsal roots appeared to be at least partly dependent on external Na$^+$. In the case of piperazine, Cl$^-$ appeared to mediate the hyperpolarizing component and Na$^+$ the depolarizing one.

Different characteristics of GABA responses on lobster muscle and in the frog spinal cord

It is important to emphasize the difference between the effects of GABA on the lobster muscle and the frog cord. The differences in membrane potential response and ionic dependency in the two tissues suggest a difference in the nature of the ionophore involved in the GABA-evoked permeability change. The results obtained with piperazine indicate that it is behaving like a GABA agonist on the lobster muscle but has only weak GABA-mimetic activity on the frog spinal cord, where it is likely to act mostly via interneuronal pathways. A possible extrasynaptic action of piperazine on both lobster muscle and frog dorsal root terminals cannot, of course, be excluded. Since piperazine only mimics the action of GABA on lobster muscle, it is tempting to suggest that the GABA receptor binding site(s) in the lobster and in the frog are different. However, this interpretation should only be considered as tentative. If a potent competitive GABA antagonist existed, then a comparison of pA_x values (Schild, 1947) using GABA and piperazine as agonists would be of great value in clarifying this matter. Unfortunately, no sufficiently potent competitive antagonist of GABA on these preparations is yet available and the classical irreversible antagonist of monoamine receptors and cholinoceptors, dibenamine (Furchgott, 1966) proved to be ineffective in suppressing the responses to GABA in both preparations.

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A COMPARATIVE STUDY OF THE EFFECTS OF GLUTAMATE AND KAINATE ON THE LOBSTER MUSCLE FIBRE AND THE FROG SPINAL CORD

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1 The depolarizing actions of glutamate and its conformationally restricted analogue kainate were investigated on the lobster muscle fibre and the frog spinal cord using intracellular and extracellular recordings, respectively.

2 Bath-applied kainate was less potent than glutamate on the lobster fibre but more potent on the frog cord. From the log–log transformation of dose-response curves it was proposed that more than one glutamate molecule was necessary to activate both the lobster and the frog receptor sites. In the frog, at least three kainate molecules were thought to be required for receptor activation.

3 The ionic dependence of glutamate and kainate responses appeared different for the two tissues.

4 Some possible explanations of the differential tissue sensitivity to kainate are discussed.

Introduction

Glutamic acid is an acidic amino acid present in high concentrations in nervous tissue and able to produce neuronal excitation when applied exogenously; a neurotransmitter role for this substance is considered likely (Johnson, 1972; Curtis & Johnston, 1974). However, a better understanding of the function of this amino acid is hampered by the lack of specific antagonists and the scarcity of quantitative studies. For this reason little is known about glutamate receptors although binding of this substance to rat brain synaptic membranes (Roberts, 1974) and purified proteins of crustacean muscle (Fiszer de Plazas & de Robertis, 1974) has been described. Kainate, an amino acid structurally related to glutamate, has recently been found to be more potent than glutamate in depolarizing vertebrate central neurones (Shinozaki & Konishi, 1970; Johnston, Curtis, Davies & McCulloch, 1974; McCulloch, Johnston, Game & Curtis, 1974; Biscoe, Evans, Headley, Martin & Watkins, 1975). In contrast, kainate has a weaker depolarizing action than glutamate at the crayfish (Shinozaki & Shibuya, 1974; Takeuchi & Onodera, 1975) and locust (Clements & May, 1974) neuromuscular junctions.

Prompted by these reports on the differing sensitivities of vertebrate and invertebrate tissues to kainate, we attempted a quantitative comparison of the dose-response relationships to bath applied glutamate and kainate on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord. In both these preparations glutamate is considered to be a putative excitatory transmitter (Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970; Barker, Nicoll & Padjen, 1975). The ionic dependence of the amino acid-evoked depolarizations in both tissues was also investigated. Some of our results have been published in preliminary form (Constanti & Nistri, 1975).

Methods

Lobster muscle fibre

Claw opener muscles of the first or second walking leg of the lobster (*Homarus vulgaris*) were dissected and prepared for intracellular recording as described in the preceding paper (Constanti & Nistri, 1976). Single superficial muscle fibres were impaled with two glass microelectrodes placed within 50 µm of each other at the centre of the fibre. One electrode filled with 2 M tripotassium citrate recorded the membrane potential while the other filled with 0.6 M potassium sulphate passed constant hyperpolarizing current pulses through the membrane. The resultant electrotonic potentials were recorded as previously described (Constanti & Nistri, 1976).

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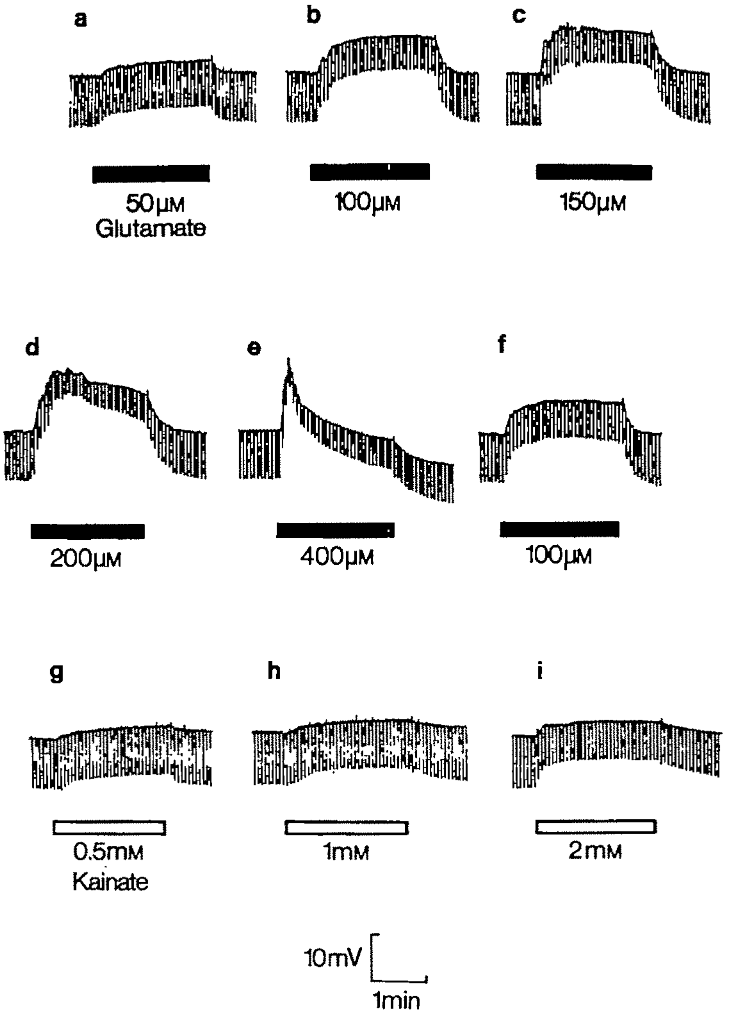

Figure 1 Depolarizations evoked by L-glutamate (filled bars) and kainate (open bars) recorded at the centre of a single lobster muscle fibre. Downward deflections are electrotonic potentials resulting from the application of control intracellular current pulses (800 ms; 2.5×10^{-7} A). (a–e) Show the effects of successively increasing concentrations of glutamate (50 μM to 400 μM); (f) is a recovery response. Each response was separated by at least 5 min washing in drug-free solution; (g–i) show the effects of kainate (0.5 mM to 2 mM). Note the relatively weaker effect of kainate and the rapidly waning effect of glutamate at high concentrations (d and e). All measurements were made on the same fibre (Resting potential = −76 mV).

Frog spinal cord

The method for setting-up and recording from the frog (*Rana temporaria*) isolated spinal cord is given in the preceding paper (Constanti & Nistri, 1976).

Drugs

Glutamate was purchased from Sigma and kainate from Calbiochem.

Results

Lobster muscle fibre

When glutamate (50 μM to 400 μM) was added to the superfusion fluid, a depolarization of the muscle membrane was produced (2–18 mV). This effect was reproducible and had a relatively fast onset and decline rate (Figure 1, a–f). At concentrations higher than 150 μM glutamate often caused a contraction of

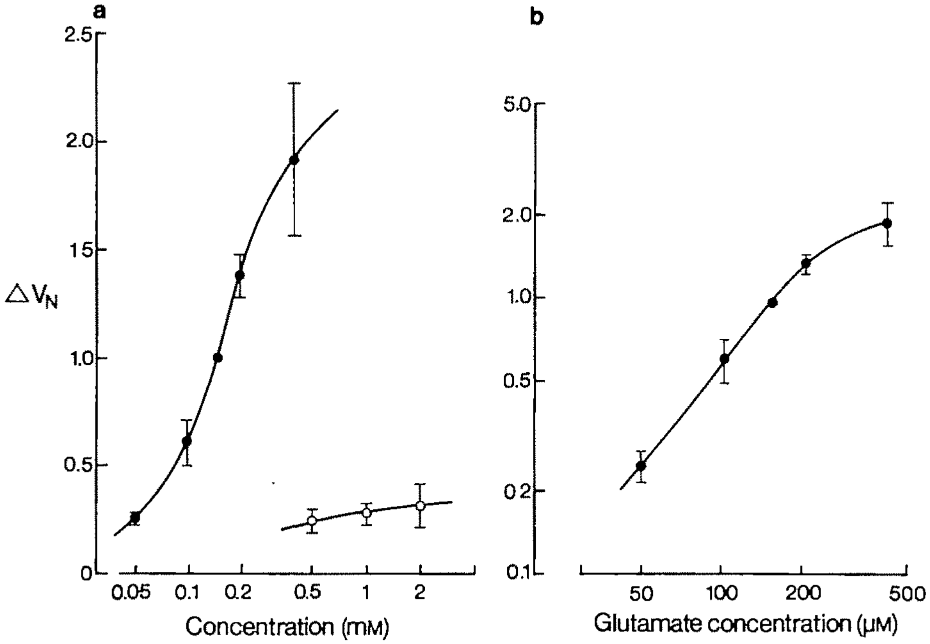

Figure 2 (a) Normalized log dose-response curves for glutamate (●) and kainate (O) in single lobster muscle fibres. Points represent mean of 3 experiments. Vertical lines show s.e. mean. Ordinate scale represents normalized membrane depolarization (ΔV_N); abscissa scale gives concentration of amino acid added to the superfusing solution. ΔV_N was calculated by dividing all glutamate and kainate depolarizations of any single fibre by the response to 150 μM glutamate (approximately half maximal) in that fibre. This allowed data from different muscle fibres to be represented on the same graph. Note that the kainate dose-response curve was almost flat. (b) Log ΔV_N vs. log [glutamate] transformation of the glutamate dose-response curve of Figure 2a. The limiting slope (in the concentration range studied) was 1.24. Error intervals indicated are the transformed standard errors.

the fibre with consequent displacement of the microelectrodes. Attempts to prevent this effect by pretreatment of the muscle with 400 mM glycerol were unsuccessful. However, in a number of preparations in which contractions following high doses of glutamate did not occur, the glutamate-induced depolarization was quite large and tended to fade away despite the continued presence of the drug (Figure 1e). Such an effect is likely to be the result of receptor desensitization (Takeuchi & Takeuchi, 1964). Where possible, log dose-response curves to the effect of glutamate were obtained (Figure 2a), but definite maxima were not attained because of desensitization and the risk of muscle contraction. Where necessary, the glutamate depolarizations were corrected to account for the slight non-linearity in the current/voltage relationship in the depolarizing direction.

The effect of kainate (0.5 mM to 2 mM) on the lobster muscle fibres was different from that of glutamate. Even though both compounds were able to depolarize the preparation, kainate was much less potent than glutamate on a molar basis (Figure 1, g–i) and the kainate dose-response curve was almost flat (Figure 2a). The decline in the effect of kainate was also somewhat slower than that of glutamate and no desensitization to kainate was seen in the concentration range investigated. Since the maximal effect of glutamate was not available, the conventional Hill plot could not be used to study the kinetics of drug-receptor interaction. Instead, the log–log plot (Werman, 1969) was employed in order to estimate the number of glutamate molecules interacting with a single receptor site, bearing in mind that depolarizations rather than conductance changes were being measured in these experiments. Figure 2b shows that in the case of glutamate the plot was linear in the initial portion and had a limiting slope (estimated from the lower two points) of 1.24. This indicates that more than one molecule of glutamate was interacting with the receptor site (see also Dudel, 1975). A similar plot for kainate could not be obtained owing to the flat nature of the dose-response curve.

Owing to the weak depolarizing action of bath-applied kainate on invertebrate muscle (Shinozaki &

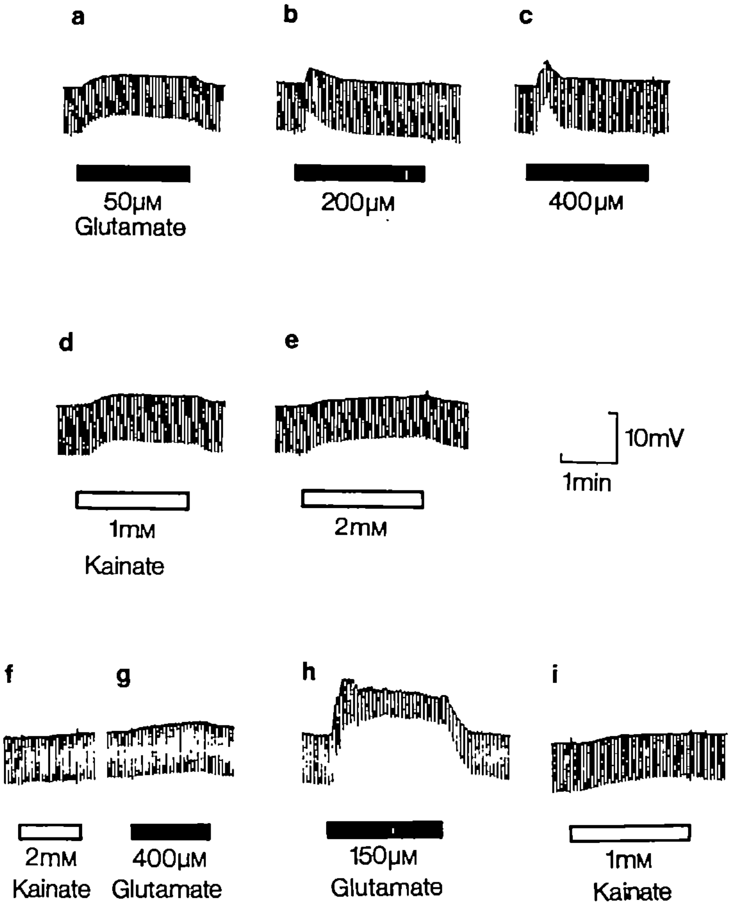

Figure 3 Effect of Na^+ and Ca^{2+} removal on glutamate and kainate depolarizations of lobster muscle fibres (recording continued from that in Figure 1); (a), (b) and (c) show responses to glutamate (50, 200 and 400 µM respectively) applied after 15 min exposure to Na^+-free (Li^+ containing) solution; (d) and (e) show responses to kainate (1 and 2 mM) in the same medium. Note that kainate responses were hardly affected (cf Figure 1h, i) whereas glutamate responses were drastically reduced although not abolished. Residual effects of kainate (2 mM) and glutamate (400 µM) were subsequently reduced after 15 min exposure to Na^+-free, Ca^{2+}-free (Mg^{2+} containing) solution (f and g); (h) and (i) show recovery responses to kainate and glutamate recorded 15 min after return to normal solution.

Shibuya, 1974; Clements & May, 1974) the ionic dependence of this amino acid depolarization has not been investigated. According to Takeuchi & Onodera (1973) the glutamate-evoked depolarization of crayfish muscle is largely dependent upon an increase in Na^+ permeability with a possible contribution from Ca^{2+}. Some experiments were therefore carried out in which the Na^+ content of the lobster saline was replaced with an equimolar amount of Li^+ (the solution was adjusted to pH 7.6 with LiOH). On changing to a Na^+-free medium there was usually a small depolarization (about 2 mV) of the muscle fibre but little change in membrane resistance. After 15 min exposure to this medium the effect of glutamate (prepared in the Na^+-free solution) was drastically reduced (Figure 3, a–c); however, a rapidly fading component of the response to glutamate was still present. The application of glutamate after 5, 20 or 60 min exposure to Na^+-free solution produced similar responses, ruling out the possibility of an insufficient removal of external Na^+ being responsible for the residual glutamate component. It was also interesting to note that small doses of glutamate produced slightly larger responses in Na^+-free medium than in a normal saline solution. In contrast, the action of kainate in Na^+-free solution was practically unchanged (Figure 3, d–e). If Ca^{2+} was also removed and substituted with an equimolar amount of Mg^{2+}, a slight depolarization

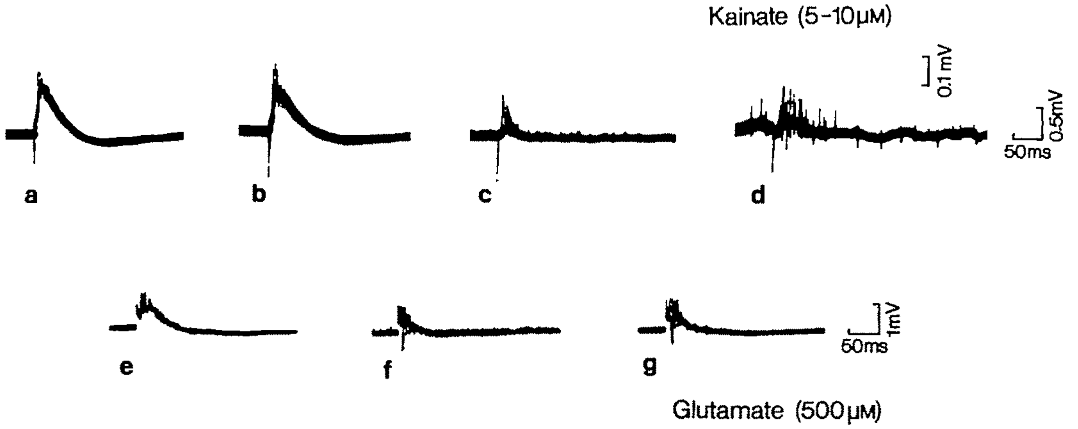

Figure 4 Effects of glutamate or kainate on the ventral root potential (VRP) of the frog isolated spinal cord; (a) and (e) controls; (b) kainate (5 µM) 2 min; (c) kainate (10 µM) 2 min; (d) kainate (10 µM) 4 min, note the higher gain; (f) and (g) glutamate (500 µM) 2 and 4 min respectively. Calibration bars for (a), (b), (c) and (d) 0.5 mV (except for (d) where a 0.1 mV bar is shown); 50 ms. Calibration bars for (e), (f) and (g) 1 mV; 50 ms.

(about 3 mV) of the membrane was produced and the effects of kainate or glutamate (residual) were virtually abolished (Figure 3, f–g). Changing from a normal solution to one containing an additional 21 mM $MgCl_2$ produced a small increase (about 5%) in the membrane resistance (see Takeuchi & Takeuchi, 1971) and also a slight increase in the amplitude of glutamate or kainate-evoked responses. All these actions were easily reversible on returning to normal lobster Ringer. Unfortunately, more detailed studies of the role of Ca^{2+} in these processes were not possible using the present method since the absence of this cation from the bathing solution depolarized the membrane and markedly reduced the membrane resistance (Takeuchi &Takeuchi, 1971) thus preventing a clear interpretation of the data.

Frog spinal cord

In the *in vitro* frog spinal cord both glutamate and kainate produced a depolarization of the ventral and dorsal roots accompanied by intense spike activity. The ventral root potential (VRP) was reduced by the bath application of kainate or glutamate and eventually abolished (Figure 4). A depression of the dorsal root potential (DRP) was also seen. Both these effects were fully reversible on washing. A feature of all these experiments was the higher potency of kainate compared to glutamate on a molar basis. Since glutamate is considered to be an excitatory transmitter of the afferents to spinal motoneurones (Barker *et al.*, 1975), it was necessary to determine whether this substance was acting directly on the motoneurones or indirectly via interneurones. In order to prevent any indirect action as a result of propagated interneuronal activity, the spinal cord was treated with tetrodotoxin (TTX; 1 µg/ml); therefore the d.c. recordings from the ventral root only represented the electrotonic spreading of the depolarization of the motoneurones along their axons (Biscoe *et al.*, 1975).

In the TTX-treated cords the ventral root depolarization produced by glutamate was dose-related, had a fast onset and decline and was often followed by a hyperpolarization (see inset of Figure 5). However, the maximum of the dose-response curve was difficult to obtain in those cases where high doses of glutamate produced a desensitization. This is reflected in Figure 5 by the scattering of the points at doses higher than 8 mM. The glutamate dose-response curve obtained in the absence of TTX was found to be very similar to that obtained in the presence of this toxin. The cord responses to glutamate could be closely reproduced even after many hours provided that the contact time of the drug was not longer than 1 min and 8–10 min elapsed between each administration. Strychnine (10 µM) did not reduce the effects of glutamate, while picrotoxin (10 µM) slightly potentiated the response to glutamate.

Figure 6 (inset) shows the ventral root responses to the application of kainate in a TTX-treated cord. This compound was active at concentrations much lower than those of glutamate and had a relatively slower onset-decline in its rate of action. The recovery of the preparation after a single administration of kainate took 12–20 min of repetitive washing; however, the addition of high doses of kainate (> 100 µM) often produced an irreversible depolarization of the cord followed by an insensitivity to glutamate and other neurally active compounds. Provided that kainate was administered at intervals of 15–20 min and doses

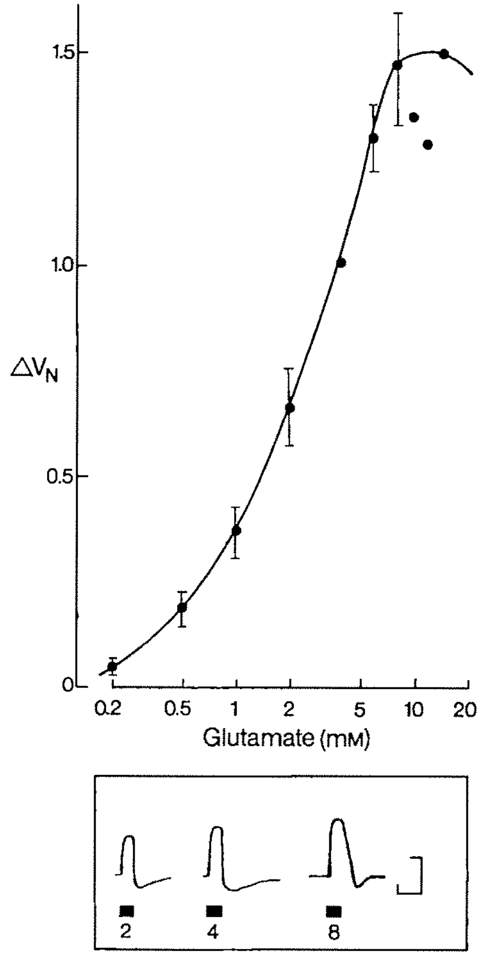

Figure 5 Normalized log dose-response curve for glutamate obtained from a ventral root of tetrodotoxin-treated spinal cords. Points represent mean of 6 experiments. Vertical lines show s.e. mean. Ordinate scale represents normalized cord depolarizations (ΔV_N) which were calculated by dividing the glutamate responses of each cord by the response to 4 mM glutamate of that cord. Abscissa scale: log concentration of glutamate. Inset: examples of cord responses to glutamate (2, 4 or 8 mM). In this and the following figures depolarizations are indicated by upward deflections of the pen. Calibration bars: 1 mV; 1 min.

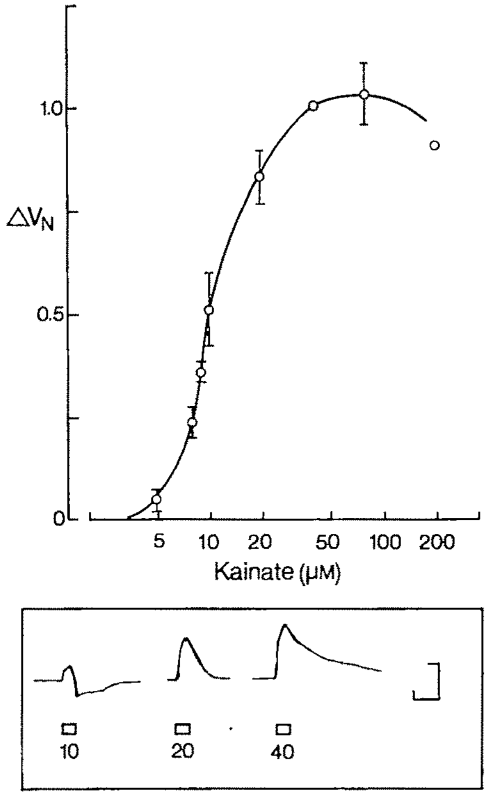

Figure 6 Normalized log dose-response curve for kainate obtained from a ventral root of tetrodotoxin-treated spinal cords. Points represent mean of 4 experiments. Vertical lines show s.e. mean. Ordinate scale represents normalized cord responses (ΔV_N) which were calculated by dividing the kainate responses of each cord by the response to 40 µM kainate of that cord. Abscissa scale: log concentration of kainate. Inset: examples of cord responses to kainate (10, 20 or 40 µM). Calibration bars: 1 mV; 1 min.

higher than 100 µM were avoided, reliable dose-response curves could be obtained as in Figure 6. Of course, the maximum shown in this figure was largely influenced by the desensitization phenomenon and the irreversible effects of large doses. Similar kainate dose-response curves were obtained from cords not treated with TTX.

In order to investigate the kinetics of the drug receptor interaction, log–log plots for glutamate and kainate were prepared (Figure 7a and b). For both glutamate and kainate the plots were linear initially with limiting slopes of 1.45 and 3.33, respectively. This suggests that more than one molecule of these drugs was interacting with a single receptor site. The ionic dependence of the amino acid depolarizations was then studied by substituting over 90% of external Na^+ in the bathing solution with equimolar Li^+. After 90 min in low-Na^+ medium, the responses to glutamate and kainate were almost completely blocked; recovery was achieved 60 min after return to the normal salt solution (Figure 8). The removal of Ca^{2+} and its replacement with equimolar Mg^{2+} did not affect the depolarization produced by glutamate but increased the subsequent hyperpolarization (Figure 8).

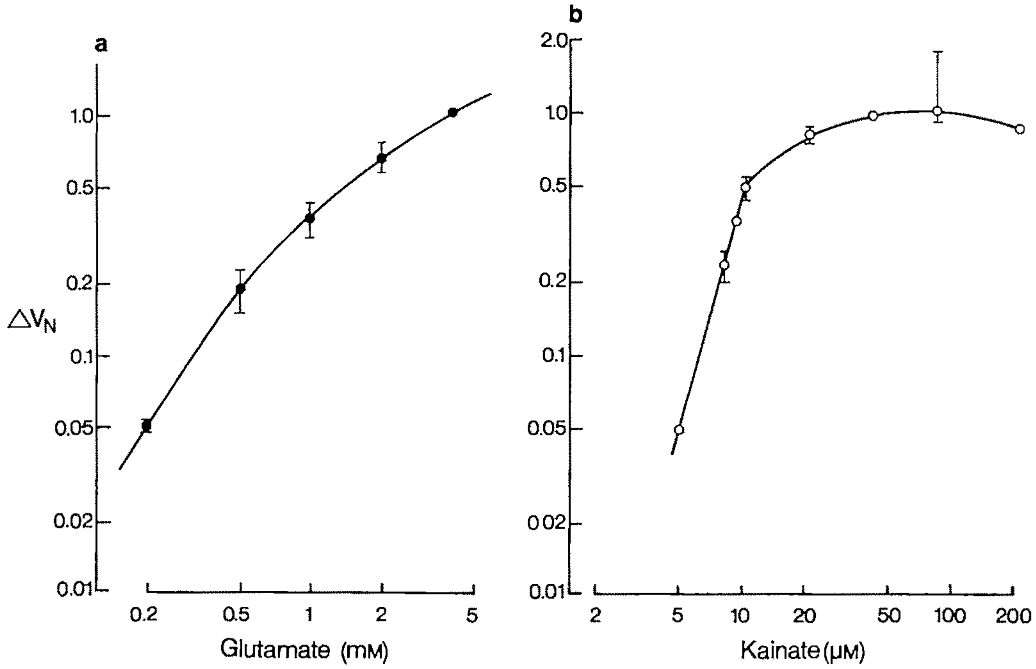

Figure 7 Log–log transformations of the dose-response curves for glutamate (●) and kainate (○) from a ventral root of tetrodotoxin-treated spinal cords; (a) Log ΔV_N vs log [glutamate], slope = 1.45; (b) Log ΔV_N vs log [kainate], slope = 3.33. Error intervals indicated are the transformed standard errors. For further details see Figures 5 and 6.

Similar results were obtained with kainate in a Ca^{2+}-free solution.

Discussion

A quantitative study of the effects of glutamate and related compounds on nervous tissue preparations suffers from several technical limitations. If this substance is applied microiontophoretically almost all neurones are excited. This widespread action complicates the assessment of glutamate as a putative neurotransmitter. Moreover, dose-response curves are difficult to obtain by iontophoretic administration (Curtis, Duggan & Johnston, 1971) despite the different methods proposed (Hill & Simmonds, 1973; Clarke, Forrester & Straughan, 1974). In order to avoid these difficulties and to allow the construction of dose-response curves, we carried out some quantitative experiments on two *in vitro* preparations, the lobster muscle fibre and the frog spinal cord. In both these tissues glutamate and kainate, a structurally related amino acid, were applied via the bathing solutions.

On the lobster muscle fibre where glutamate is considered to be the excitatory transmitter (Kravitz *et al.*, 1970) this substance had a depolarizing effect which showed rapid desensitization at high concentrations (if muscle contraction did not occur). The glutamate-evoked depolarization was largely dependent on external Na^+ (see Gerschenfeld, 1973, for review) and required more than one molecule for the receptor activation. However, it should be emphasized that the log–log plot analysis used in this study can give an underestimate of the true degree of cooperativity when the true limiting slope (attained as glutamate concentration approaches zero) lies outside the physiological range of the measurements.

In contrast, kainate showed a weaker depolarizing effect than glutamate (on a molar basis) which was relatively unaltered in the presence of a Na^+-free solution, but was abolished in a Na^+ and Ca^{2+}-free solution. In spite of the limitations of the Ca^{2+}-free experiments, a role for this divalent cation in the muscle responses to glutamate and kainate can thus be envisaged and is supported by the findings on other crustacean muscle preparations (Takeuchi & Onodera, 1973).

In the frog spinal cord both glutamate and kainate produced dorsal and ventral root depolarization, intense spike activity and depression of the VRP and DRP presumably as a consequence of excessive

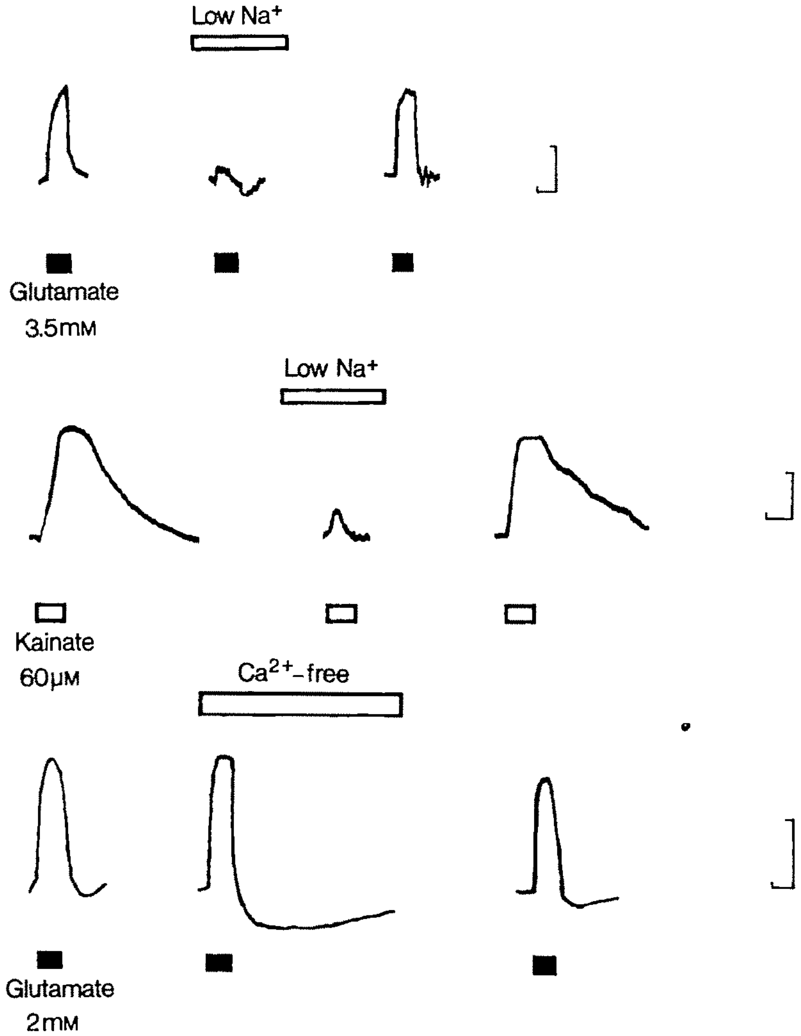

Figure 8 Effects of ionic changes on glutamate and kainate-evoked depolarizations recorded from ventral roots in tetrodotoxin-treated spinal cords. The first response in each row is a control and the last is a recovery (60 min after low Na^+ Ringer and 30 min after Ca^{2+}-free Ringer respectively). The centre responses were obtained after either 90 min in low Na^+ (replaced by equimolar Li^+) medium or 30 min in Ca^{2+}-free (replaced by equimolar Mg^{2+}) medium. Drugs were applied as indicated by the filled bars (glutamate) or open bars (kainate) below the traces. All calibrations: 1 mV; 1 min.

depolarization (Curtis, Phillis & Watkins, 1961). The glutamate dose-response curves obtained in TTX-treated cords, using the depolarization recorded from the ventral root as the response parameter, were similar in shape to those obtained in the absence of this toxin. The same was also found for kainate. This suggests that both drugs were acting directly on the motoneurones. A drug-induced depolarization of the afferent synaptic knobs with consequent discharge of transmitters onto the motoneurones seems unlikely since the glutamate or kainate-induced depolarization was essentially unchanged in Ca^{2+}-free Ringer (containing Mg^{2+}) in which synaptic transmission was blocked. However, a contribution of increased extracellular K^+ to the observed motoneuronal depolarization following the administration of these amino acids cannot be excluded.

In all our experiments on the frog cord, kainate was more potent than glutamate (on a molar basis), thus confirming previous observations on the vertebrate central nervous system (Shinozaki & Konishi, 1970; Johnston *et al.*, 1974; McCulloch *et al.*, 1974; Biscoe *et al.*, 1975). However, the action of kainate had a slower onset and decline relative to glutamate and was irreversible at high concentrations. The latter finding might explain the considerable *in vivo* neurotoxicity of this drug (Olney, Rhee & Ho, 1974). From the analysis of our results it would appear that glutamate and kainate interact with the frog motoneuronal membrane via a Na^+-dependent mechanism. Moreover, more than one molecule of glutamate seems to be involved in the receptor interaction, while in the case of kainate a number even higher than this can be envisaged. Kainate may thus be considered to act as an agonist on glutamate receptors in this system, as has been suggested in the mammalian central nervous system (Johnston *et al.*, 1974; McCulloch *et al.*, 1974).

If kainate was also acting on glutamate receptors in lobster muscle, then the observed difference in tissue sensitivity to this agent might be explained if the frog spinal glutamate receptors had a relatively higher affinity for kainate. An essentially similar glutamate binding site on both preparations could thus show a different tolerance to variations in agonist structure, the invertebrate site being the more restricted in this respect. A difference in the diffusion rate of kainate through the two tissues could be excluded since, on the lobster muscle where kainate was less potent, only single superficial fibres were used for recording, and these were in direct contact with the superfusing fluid and therefore more accessible to this drug than spinal motoneurones.

At the crayfish neuromuscular junction, both junctional and extrajunctional glutamate receptors have been postulated (Shinozaki & Shibuya, 1974) although only the extrajunctional receptors are thought to be sensitive to kainate (Takeuchi & Onodera, 1975). It is possible, therefore, that kainate was acting on extrajunctional glutamate receptors in both lobster muscle and the frog spinal cord. The different tissue sensitivity to kainate would then be explained if the two preparations contained a different number of such receptors. However, evidence of spinal extrajunctional glutamate receptors is not yet available due to the lack of suitable techniques and the presence of extrajunctional glutamate receptors on lobster muscle needs to be tested experimentally.

In conclusion, our present findings suggest that the receptor sites activated by glutamate in the lobster and the frog do not constitute a homogeneous population. In the absence of specific glutamate antagonists (Nistri & Constanti, 1975), the use of kainate together with other glutamate agonists may be a useful tool with which to characterize these receptor sites.

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THE CONTRIBUTION OF EXTRANEURONAL UPTAKE TO THE TRACHEA-BLOOD VESSEL SELECTIVITY OF β-ADRENOCEPTOR STIMULANTS *in vitro* IN GUINEA-PIGS

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1 The potencies relative to isoprenaline of isoetharine, tertiary butyl noradrenaline, salbutamol, orciprenaline, Me 506, rimiterol, fenoterol, carbuterol and terbutaline on isolated preparations of guinea-pig trachea and blood vessels (perfused hind limb) were determined. All the compounds were selective for trachea and selectivity values, i.e. relative potency on trachea divided by relative potency on hind limb, ranged from 2.3 to 21.4.

2 Responses to isoprenaline (the reference compound), tertiary butyl noradrenaline and isoetharine were potentiated on trachea by 50 µM phenoxybenzamine (PHB) and by other inhibitors of extraneuronal uptake (ENU). Under these conditions the selectivity values of all the compounds was close to unity.

3 Selectivity values were also close to unity if they were calculated from data obtained without ENU inhibition, provided that only those compounds not potentiated by PHB on trachea were used.

4 It is proposed that the trachea-blood vessel selectivity shown by β-adrenoceptor stimulants can be caused by the influence of ENU upon them, rather than by their ability to distinguish between two β_2-adrenoceptors.

5 The suggestion that differences exist between β_2-adrenoceptors in respiratory and vascular smooth muscle is not supported by the *in vitro* experiments described.

Introduction

In a previous study the potencies relative to isoprenaline of a number of resorcinolamines were markedly higher on the isolated trachea than on the isolated perfused hind limb of guinea-pigs i.e. the compounds showed trachea/blood vessel selectivity (O'Donnell & Wanstall, 1974). Although it was tempting to suggest that this selectivity indicated a difference between adrenoceptors previously classified as being the same (β_2-adrenoceptors), other explanations for this observed selectivity, which did not require subdivision of β_2-adrenoceptors, were cited. The results described in the present paper provide support for the explanation that trachea/blood vessel selectivity *in vitro* reflects an influence of extraneuronal uptake on the potency of compounds rather than an ability of compounds to distinguish between two receptor types.

Methods

Isolated tracheal chain and perfused hind limb preparations

Tracheal chain preparations (relaxation) and isolated perfused hind limb preparations (vasodilatation) were set up as described by O'Donnell and Wanstall (1974). On each preparation maximum responses to isoprenaline were obtained and responses expressed as a percentage of that maximum.

Inhibition of extraneuronal uptake

(i) *On trachea.* The inhibitors of extraneuronal uptake tested were hydrocortisone (50 µM), deoxycorticosterone acetate (DOCA, 5 µM and 20 µM), metanephrine (10 µM and 100 µM) and phenoxybenzamine (PHB, 1 µM, 10 µM, 50 µM and 100 µM). Hydrocortisone, DOCA or metanephrine was added to the Krebs solution bathing the tissue 30 min before the start of the estimation of a concentration-response line and remained in contact with the tissue whilst the concentration-response line was obtained. Phenoxybenzamine was in contact with the tissue for 15, 30, 60 or 90 minutes. The tissue was then washed thoroughly for 10 min and the subsequent concentration-response line obtained in PHB-free Krebs solution.

(ii) *On hind limb.* Hind limb preparations were examined after perfusion with Krebs solution containing PHB (10 µM and 100 µM), hydrocortisone (10 µM) or metanephrine (10 µM). Perfusion times varied from 10 to 40 min in different experiments. After PHB treatment, but not after hydrocortisone and metanephrine, perfusion was carried out in drug-free Krebs solution.

Treatment of data

(i) *Relative Potency.* Concentration-response or dose-response lines to isoprenaline and to at least one test compound were obtained on each of several preparations, using a randomized order of addition of the compounds. The log EC_{50} or ED_{50} (concentration or dose producing 50% maximum response) was then interpolated for the test compound and for isoprenaline. The potency of the test compound relative to that of isoprenaline (assigned a value of 100) was then calculated from the following formula:

$$\text{Relative potency} = 100 \times \text{antilog } [\text{mean } (\text{neg log } EC_{50_{test}} - \text{neg log } EC_{50_{iso}})]$$

(ii) *Selectivity for trachea.* The relative potency value on the trachea was divided by that on the hind limb blood vessels. Compounds showing selectivity for the trachea gave high values, but a selectivity value approaching 1 indicated selectivity for neither tissue.
(iii) *Potentiation by phenoxybenzamine.* Concentration-response lines to each compound were obtained before and after treatment with phenoxybenzamine. From a minimum of 4 experiments a value for potentiation was obtained from the formula:

$$\text{Potentiation} = \text{mean } (\text{neg log } EC_{50_{after\ PHB}} - \text{neg log } EC_{50_{before\ PHB}})$$

The antilog of this mean value gave the factor by which the EC_{50} was reduced after PHB treatment.

Drugs

The β-adrenoceptor stimulants examined were: carbuterol hydrochloride (Smith, Kline & French); fenoterol hydrobromide (Th1165a, Boehringer-Ingelheim); isoetharine hydrochloride (Sterling-Winthrop); (±)-isoprenaline sulphate (Burroughs Wellcome); Me 506 hydrobromide (Boehringer-Ingelheim); orciprenaline sulphate (Boehringer-Ingelheim); rimiterol hydrobromide (Riker); salbutamol base (Allen and Hanbury); terbutaline hydrochloride (Me 501, Boehringer-Ingelheim); terbutaline sulphate (Astra); (±)-tertiary butyl noradrenaline (Sterling-Winthrop). These drugs were donated by the various companies named.

Other drugs used were: deoxycorticosterone acetate (BDH); hydrocortisone hemisuccinate sodium (Glaxo); metanephrine hydrochloride (Calbiochem); phenoxybenzamine hydrochloride (Smith, Kline & French).

All drugs were obtained as pure powders. The β-adrenoceptor stimulants were made up in 0.01 N HCl to give stock solutions of 10 mM. Dilutions were made in Krebs solution containing ascorbic acid (0.2 μg/ml) and kept on ice for the duration of each experiment. Phenoxybenzamine was dissolved in 95% ethanol containing 0.001 ml 10 N HCl/ml to give a 100 mM stock solution. Dilutions were made in Krebs solution.

Statistical analyses

The measure of variation of the mean quoted is the standard error (s.e.). Mean relative potency values are quoted with 95% confidence limits. A paired *t*-test was used to assess the significance of the difference between paired log EC_{50} values.

Results

Inhibition of extraneuronal uptake (ENU)

Of the ENU inhibitors tried, phenoxybenzamine was the only drug which did not cause marked relaxation of tracheal preparations. The responses to isoprenaline on trachea were potentiated after 30 min contact of the tissue with 10 μM, 50 μM or 100 μM PHB but not after 1 μM. The potentiation was the same after either 50 μM or 100 μM and maximum potentiation was achieved after 30 minutes. Thus 50 μM PHB for 30 min was used in the tracheal experiments. The potentiation of isoprenaline produced by this treatment was maintained unchanged for several hours after removal of free PHB. Responses to seven of the β-adrenoceptor stimulants examined were not significantly affected by PHB whereas responses to the other three compounds were significantly potentiated (Table 1) i.e. isoprenaline (5.2-fold), tertiary butyl noradrenaline (2.3-fold) and isoetharine (2.0-fold). This could be taken as evidence that the pharmacological responses to three compounds used in this study are modified by loss into ENU sites.

In only 4 of the experiments with other ENU inhibitors was the relaxation produced by the inhibitor small enough to allow concentration-response lines to isoprenaline to be obtained. In these 4 experiments a potentiation of isoprenaline was observed of similar magnitude to that produced by PHB i.e. 6.8-fold with hydrocortisone (50 μM), 4.4-fold with DOCA (5 μM), 5.5-fold with DOCA (20 μM) and 4.8-fold with metanephrine (10 μM). This potentiation was not maintained when the inhibitor was washed out.

No data on hind limb preparations treated with ENU inhibitors were obtained since preparations became unresponsive after perfusion with the ENU inhibitors tried.

Selectivity for trachea

In the initial series of experiments an ENU inhibitor was not used and, for each of the test compounds, the relative potency value on trachea was higher than that on the hind limb i.e. they all had selectivity values greater than 1. Six of the test compounds had values greater than 10 (Table 2, Column 4). Since the responses of the trachea to the reference compound isoprenaline, as well as to two of the test compounds, had been shown to be modified by PHB, all relative potency values on trachea (and hence all selectivity values) would be altered if the influence of ENU was excluded. Calculation of selectivity values using data obtained from experiments in which an ENU inhibitor was present was not possible because no data could be obtained on hind limb preparations after ENU inhibitors. An alternative approach was to use the original data but to omit from the analysis those compounds whose responses were modified by ENU (i.e. potentiated by PHB) i.e. isoetharine, tertiary butyl noradrenaline and isoprenaline. This approach required the replacement of isoprenaline by another reference compound. When relative potency and selectivity values of the remaining seven compounds were recalculated with respect to any of these seven compounds as 100 on both tissues, the relative potency values on trachea and hind limb were similar and selectivity values were not sufficiently different from unity to suggest different receptors in the two tissues. Table 3 illustrates such a calculation using orciprenaline as 100 on both tissues. It can be seen that the selectivity values lie within the range 0.4 to 2.1 compared to the range of 2.3 to 21.4 shown in Table 2 when isoprenaline is the reference compound.

It is interesting to note that the relative potency values obtained on trachea in the series of experiments in which ENU was inhibited by phenoxybenzamine (Table 2, Column 2) were very similar to those obtained on hind limb preparations in which ENU had not been inhibited (Table 2, Column 3). If these relative potency values were used to calculate selectivity, values close to unity resulted (Table 2, Column 5) resembling those obtained in Table 3.

Discussion

A difference between the relative potency values of a series of agonist or antagonist compounds on two tissues is frequently taken as one line of evidence for the receptors in those two tissues being different. Furchgott (1972) has defined a set of optimal conditions which should be met before

Table 1 Potentiation of β-adrenoceptor stimulants by phenoxybenzamine (PHB) (50 μM for 30 min, then PHB-free Krebs) on trachea

Compound	*Mean potentiation* ± s.e.*	*Antilog potentiation*
Isoprenaline	0.72 ± 0.04‖ (27)†	5.2
Tertiary butyl noradrenaline	0.36 ± 0.05§ (5)	2.3
Isoetharine	0.31 ± 0.08‡ (5)	2.0
Orciprenaline	0.08 ± 0.10 (5)	1.2
Salbutamol	0.07 ± 0.12 (4)	1.2
Terbutaline	0.06 ± 0.12 (4)	1.1
Carbuterol	0.05 ± 0.07 (4)	1.1
Fenoterol	–0.02 ± 0.08 (5)	0.96
Rimiterol	–0.11 ± 0.10 (4)	0.78
Me 506	–0.14 ± 0.08 (4)	0.72

* mean [neg log $EC_{50\ \text{after PHB}}$ – neg log $EC_{50\ \text{before PHB}}$]; † number of paired observations; ‡ $0.05 > P > 0.01$; § $0.01 > P > 0.001$; ‖ $P < 0.001$ (paired t test in all cases).

pharmacological observations, such as relative potency values of agonists, are used as the basis for the, characterization of receptors. In studies involving sympathomimetic amines, optimal conditions would not exist if loss of compounds into neuronal and/or extraneuronal uptake sites is influencing the concentration in the receptor biophase. Several workers have reported that the potencies of certain compounds, as either β-adrenoceptor agonists or antagonists, were different in respiratory and vascular smooth muscle i.e. the compounds were selective. From this they have suggested that differences exist between β-adrenoceptors in these two tissues (Bristow, Sherrod & Green, 1970; Wardell, Colella, Shetzline & Fowler, 1974; Wasserman & Levy, 1974). Although isoprenaline is used as the reference compound in these and most other studies on β-adrenoceptors and is known to have an affinity for extraneuronal uptake (ENU), the influence of ENU is rarely excluded. In our previous study (O'Donnell & Wanstall, 1974) ENU was not inhibited and therefore, although we observed a 10 to 20-fold difference between the relative potencies of some resorcinolamines on tracheal and perfused hind limb preparations, this could not be taken as evidence that the β-adrenoceptors in trachea and hind limb blood vessels were different. In the present study the differences between the relative potencies of an extended series of compounds (and hence their trachea/blood vessel selectivity) disappeared when the influence of ENU was excluded. This suggested that the observed

Table 3 Relative potencies and selectivity for trachea of those β-adrenoceptor stimulants which were not potentiated by phenoxybenzamine.

	*Relative potency**		
Compound	*Trachea*	*Hind Limb*	*Selectivity*
Orciprenaline	100	100	1.0
Salbutamol	500	1230	0.4
Me 506	739	686	1.1
Rimiterol	1045	933	1.1
Fenoterol	3540	3010	1.2
Carbuterol	274	171	1.6
Terbutaline	274	133	2.1

* Results from Table 2 (Cols 1 and 3) recalculated with orciprenaline as reference compound (assigned a value of 100).

Table 2 Relative potencies and selectivity for trachea of β-adrenoceptor stimulants on trachea and on hind limb blood vessels.

	Relative potencies			*Selectivity for trachea (T÷BV)*	
	Trachea (T)		*Hind limb blood vessels (BV)*		
Compound	*No PHB*	*PHB*		*Using T (no PHB)*	*Using T (PHB)*
Isoprenaline*	100	100	100	1.0	1.0
Isoetharine	52.5(5)† (32–87)‡	18.6(5) (15–28)	22.9(5) (18–30)	2.3	0.8
Tertiary butyl noradrenaline	468(5) (251–871)	155(5) (79–302)	178(3) (66–479)	2.6	0.9
Salbutamol	55.0(5) (22–130)	14.5(5) (6–38)	12.9(5) (7–23)	4.3	1.1
Orciprenaline	11.0(5) (5–22)	1.91(5) (0.9–4.1)	1.05(5) (0.61–1.8)	10.5	1.8
Me 506	81.3(6) (43–155)	7.08(4) (5.6–8.9)	7.24(3) (2.2–24)	11.3	1.0
Rimiterol	115(5) (50–263)	6.76(5) (3–16)	9.77(5) (6–16)	11.8	0.7
Fenoterol	389(6) (141–1072)	60.3(6) (48–76)	31.6(5) (22–45)	12.3	1.9
Carbuterol	30.2(5) (17–54)	6.61(6) (3.3–13)	1.78(5) (1.3–2.5)	17.0	3.7
Terbutaline	30.2(5) (22–42)	3.80(5) (1.8–8.1)	1.41(5) (0.6–3.2)	21.4	2.7

* = reference compound; † = number of observations; ‡ = 95% confidence limits.

selectivity was not necessarily related to receptor differences but could be explained by implicating ENU.

An important assumption made in this paper is that the potentiation of isoprenaline by PHB on trachea results from the inhibition of ENU. A number of observations would support this assumption. Foster (1967, 1969) showed that the potentiation of responses to isoprenaline on trachea correlated with inhibition of the uptake and retention of [^{3}H]-isoprenaline by tracheal tissue. O'Donnell & Saar (1972) have also shown, using fluorescence histochemistry, that PHB prevents extraneuronal accumulation of noradrenaline in guinea-pig trachea. Other drugs described as ENU inhibitors were shown in the present study to cause potentiation of responses to isoprenaline and the magnitudes of these potentiations were similar to those seen after PHB. The lack of potentiation of orciprenaline and other compounds used in the present study eliminates the possibility that PHB caused a non-specific sensitization of β-adrenoceptors.

If ENU is to explain the selectivity it is necessary to postulate that, in guinea-pigs, pharmacological responses to compounds which have an affinity for ENU, e.g. isoprenaline, are influenced by ENU to different extents in trachea and blood vessels. Responses to isoprenaline were modified by ENU inhibitors on trachea but data could not be obtained on hind limb preparations. Thus we have no direct evidence for a differential effect of ENU on responses in these two tissues. Nevertheless it is feasible that vascular responses in guinea-pigs are not modified by ENU inhibitors since atrial responses to isoprenaline are not modified by ENU inhibitors (unpublished observations; Woppel & Trendelenburg, 1973). Also, vascular smooth muscle of guinea-pig, unlike that of rabbit, rat and mouse, cannot accumulate and retain extraneuronal fluorescence after incubation in noradrenaline (Gillespie & Muir, 1970). Much reduced values for selectivity were obtained in the present study, not only when the influence of ENU was excluded on both tissues (by choosing the appropriate compounds) but also when it was excluded only on trachea (by using PHB to inhibit ENU). These observations could be taken as indirect evidence that ENU has little effect in modifying responses on hind limb vessels. Also, they do not support the suggestion that differences exist between β_2-adrenoceptors in respiratory and vascular smooth muscle.

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THE CHOLINERGIC COMPONENT IN THE REFLEX VASODILATATION ELICITED BY STIMULATION OF THE DEPRESSOR NERVES IN THE RABBIT

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1 The effects of the stimulation of the cephalic endings of the depressor nerve on the resistance in the perfused hindlimb were studied in the rabbit.

2 The vasodilatation thus elicited in the perfused hindlimb was reduced either by administration of guanethidine or by sympathectomy and abolished by subsequent treatment with atropine.

3 These data confirm the existence of two components in the genesis of the reflex vasodilatation: a passive component, due to the inhibition of sympathetic discharge, and an active component which in the rabbit is cholinergic in nature.

Introduction

Many investigators conclude that reflex vasodilatation results, at least partly, from an active mechanism since the magnitude of reflex vasodilatation usually exceeds the magnitude of dilatation produced by sympathectomy (Beck, 1961; Beck & Brody, 1961; Sakuma & Beck, 1961). There is a controversy regarding the nature of this active component in the dog. Some investigators suggest it is histaminergic (Beck & Brody, 1961; Beck, 1965; Tuttle, 1965; Brody, 1966; Wellens, 1968) and others suggest it is cholinergic (Rengo, Chiariello, De Caprio, Saccà, Trimarco, Perez & Condorelli, 1975). A histaminergically mediated reflex dilatation has also been observed in the cat (Tuttle, 1967), the rat (Tobia, Myia & Bousquet, 1968) and more recently in the monkey (Levin, Bartlett & Beck, 1968).

The participation of the cholinergic system in the genesis of the reflex has long been denied. However, Takeuchi & Manning (1971; 1973) recently demonstrated the existence of a cholinergic component in the genesis of reflex dilatation in the cat during sustained baroreceptor stimulation with high levels of carotid sinus perfusion pressure. Furthermore, Zanchetti & Ellison (1973) showed the possibility of a muscular cholinergic vasodilatation in the cat.

In this study we wished to demonstrate the participation of the cholinergic system in the reflex vasodilatation elicited in the hindlimb of the rabbit by stimulation of the depressor nerves, which in this animal are anatomically separate from the vagosympathetic trunks.

Methods

Male rabbits, weighing 2–3 kg, were anaesthetized with sodium thiopentone (40 mg/kg i.v.); additional doses of 6 mg/kg were given as required. The trachea was intubated and artificial ventilation was performed with O_2 at a rate of 30–36 cycles per minute.

The hindlimb was perfused at constant flow with blood of the same animal by means of a peristaltic pump (Sigmamotor T6); for this purpose, after administration of heparin (5 mg/kg) by intravenous injection, a polyethylene catheter was introduced through the external iliac artery into the abdominal aorta and the blood was pumped into the femoral artery of the same side. Under these conditions, changes in vascular tone of the perfused hindlimb were reflected by proportional changes in perfusion pressure. The rabbits were paralysed with gallamine triethiodide (3 mg/kg i.v.).

At the beginning of each experiment, blood flow was adjusted to give a perfusion pressure approximately equal to systemic blood pressure and left unchanged throughout the course of the experiment. A thermoregulated delay loop was introduced into the perfusion system so that arterial blood adrenaline arrived at the peripheral muscle after the completion of the haemodynamic response. Perfusion pressure was measured from a T connector interposed between the pump and the hindlimb, and the arterial blood pressure was recorded from a catheter placed in the thoracic aorta via the contralateral femoral artery. Battaglia-Rangoni multichannel polygraph and pressure transducers were used.

The depressor nerves and vagosympathetic trunks were exposed through an incision in the middle of the neck and then cut. A pair of Ag-AgCl electrodes was placed on the cephalic end of the depressor nerves, covered with mineral oil, and connected to an electrical stimulator (Nihon-Kohden MS 3). Only one trunk was stimulated in each experiment. The stimulation was performed with rectangular waves of 5 V, of 0.2 ms duration, and at a frequency of 20 Hz. The duration of the stimulation was 15 seconds.

In some experiments the sympathetic chain was cut at the lumbar level; in other experiments the sympathetic outflow was blocked by the intravenous administration of guanethidine (8 mg/kg). The effectiveness of the surgical or pharmacological sympathectomy was proved by the lack of change in perfusion pressure following the occlusion of the carotid arteries. The cholinergic blockade was obtained by intra-arterial administration of atropine (0.5 mg/kg). To confirm that the vascular system was reactive following pharmacological blockade induced by surgical sympathectomy or guanethidine and by atropine, the haemodynamic response to intra-arterial administration of sodium nitrite and histamine were evaluated, before and after these treatments, in five experiments.

To exclude the possibility that atropine administration could be blocking nervous transmission at a central, spinal or ganglionic level, five experiments were performed in which the reflex response was evoked by stimulation of the depressor nerve before and after intra-arterial administration of atropine (0.5 mg/kg).

The following drugs were used: sodium thiopentone (Farmotal, Farmitalia); heparin (Liquemin, Roche); guanethidine (Ismelin, Ciba); atropine sulphate (Lancellotti); histamine (Roche) (2 μg i.a.).

Statistical analysis was conducted by a paired t test (Snedecor & Cochran, 1967). All data are presented as mean ± s.e.

Results

Stimulation of the cephalic end of the depressor nerves in the control rabbits elicited a decrease in systemic blood pressure and a reflex vasodilatation in the perfused hindlimb.

Table 1 Effects of stimulation of the depressor nerves on blood pressure and perfusion pressure in hindlimb of rabbit

Treatment		*Seconds after initiation of nerve stimulation*		
		0	*24*	*48*
Stimulation alone	BP	104 ± 11	76 ± 11 $P<0.001$	97 ± 10 NS
	PP	117 ± 20	93 ± 14 $P<0.05$	107 ± 17 NS
After guanethidine (i.v.)	BP	101 ± 10	89 ± 10 $P<0.01$	95 ± 11 NS
	PP	109 ± 12	100 ± 10 $P<0.01$	109 ± 12 NS
After guanethidine plus atropine	BP	95 ± 12	88 ± 12 $P<0.001$	94 ± 11 NS
	PP	102 ± 9	102 ± 9 NS	102 ± 9 NS
Stimulation alone	BP	110 ± 8	71 ± 11 $P<0.001$	97 ± 6 NS
	PP	102 ± 12	83 ± 11 $P<0.01$	100 ± 11 NS
After sympathectomy	BP	99 ± 10	83 ± 15 $P<0.02$	100 ± 12 NS
	PP	106 ± 14	89 ± 13 $P<0.001$	102 ± 12 NS
After sympathectomy plus atropine	BP	99 ± 10	91 ± 10 $P<0.02$	85 ± 7 NS
	PP	101 ± 7	101 ± 7 NS	101 ± 7 NS

Each value represents mean ± s.e. of five animals; it is compared with the corresponding value at time zero. BP = blood pressure; PP = perfusion pressure in mmHg.

After guanethidine pretreatment or surgical sympathectomy the vasodilatation evoked by stimulation of the depressor nerve was reduced. This haemodynamic response was completely abolished by the intra-arterial administration of atropine while the behaviour of the systemic blood pressure was not significantly modified (Table 1).

On the other hand, guanethidine, sympathectomy and atropine produced no modification of the response to the intra-arterial administration of histamine and sodium nitrite (Tables 2 and 3).

Finally, the results summarized in Table 4 show that atropine alone did not abolish the reflex vasodilatation.

Discussion

Our results confirm that the reflex vasodilatation consists of two components: one passive and the other active.

The first component, that results from a decrease in sympathetic vasoconstrictor discharge, is abolished by surgical or chemical sympathectomy induced by

Table 2 Effects of intra-arterial histamine on blood pressure and perfusion pressure in hindlimb of rabbit

Treatment		*Seconds after histamine injection*		
		0	*24*	*48*
Histamine alone	BP	81± 4	81±4	81±4
	PP	90±13	113±4	108±3
			$P<0.005$	$P<0.001$
After guanethidine	BP	77± 2	77±2	77±2
	PP	94± 2	118±3	111±3
			$P<0.005$	$P<0.01$
After guanethidine plus sympathectomy	BP	75± 2	75±2	75±2
	PP	90± 4	112±3	107±3
			$P<0.001$	$P<0.001$
After guanethidine plus sympathectomy and atropine (i.a.)	BP	75± 3	75±3	75±3
	PP	88± 3	112±4	105±5
			$P<0.005$	$P<0.005$

Each value represents mean±s.e. of five animals; it is compared with the corresponding value at time zero. BP=blood pressure; PP=perfusion pressure in mmHg.

Table 3 Effects of intra-arterial injection of sodium nitrite on blood pressure and perfusion pressure in rabbit hindlimb

Treatment		*Seconds after sodium nitrite injection*		
		0	*24*	*48*
Sodium nitrite alone	BP	81±4	81±4	81± 4
	PP	87±8	68±8	74±10
			$P<0.005$	$P<0.01$
After guanethidine (i.v.)	BP	78±3	78±3	78± 3
	PP	96±4	82±4	81± 5
			$P<0.001$	$P<0.001$
After guanethidine plus sympathectomy	BP	75±3	75±3	75± 3
	PP	92±4	79±4	79± 4
			$P<0.001$	$P<0.005$
After guanethidine plus sympathectomy and atropine (i.v.)	BP	75±3	75±3	75± 3
	PP	88±4	73±4	77± 3
			$P<0.05$	$P<0.05$

Each value represents mean±s.e. of five animals; it is compared with the corresponding value at time zero. BP=blood pressure; PP=perfusion pressure in mmHg.

Table 4 Effects of stimulation of the depressor nerves on blood pressure and perfusion pressure in hindlimb of rabbit

Treatment		*Seconds after start of nerve stimulation*		
		0	*24*	*48*
Stimulation of depressor nerves	BP	98±5	81±5 $P<0.005$	92±4 $P<0.05$
	PP	100±6	67±6 $P<0.005$	90±6 $P<0.05$
Stimulation of depressor nerves after atropine	BP	97±7	82±7 $P<0.01$	90±7 NS
	PP	98±6	79±8 $P<0.01$	94±7 NS

Each value represents mean±s.e. of five animals; It is compared with the corresponding value at time zero. BP=blood pressure; PP=perfusion pressure in mmHg.

guanethidine. That the blockade of adrenergic discharge induced by guanethidine treatment was genuinely effective is demonstrated by the lack of change in perfusion pressure following section of the lumbar sympathetic chain in guanethidine-treated rabbits. On the other hand, we have demonstrated the capacity of this drug to block the sympathetic discharge in the dog (Rengo, De Caprio, Saccà, Trimarco, Perez, Chiariello & Condorelli, 1976). In this animal, guanethidine is able to reverse the vasoconstriction induced by the electrostimulation of the lumbar sympathetic chain to vasodilatation.

The active component in the rabbit is cholinergic in nature because it is abolished by atropine. This finding is very interesting since it confirms the data of Takeuchi & Manning (1971; 1973) about the existence of a cholinergic component in the genesis of the reflex dilatation, which has long been denied.

The observation that atropine alone was not able to abolish the reflex completely rules out the possibility that the dose of atropine here employed could have exerted a muscarinic block at some central or ganglionic level.

Furthermore, the observation that neither surgical sympathectomy nor administration of guanethidine or atropine induces any modification of the haemodynamic response to the intra-arterial injection of histamine and sodium nitrite proved that the vascular system was reactive following surgical or pharmacological denervation.

Regarding the neural pathway of the cholinergic innervation which mediates the active reflex vasodilatation in the rabbit, our results obtained in sympathectomized animals suggest that cholinergic fibres are distributed to the periphery through pathways other than the classical sympathetic outflow, such as the somatic nerves.

The lack of a histaminergic component in the rabbit is not in disagreement with the data of the authors who observed a histaminergically mediated reflex dilatation in several other species (Beck, 1965; Tuttle, 1965; Brody, 1966; Tuttle, 1967; Brody, 1968; Wellens, 1968) since histamine produces a hypertensive effect in the rabbit (Goth, 1968).

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UTERINE RECEPTORS FOR OXYTOCIN: CORRELATION BETWEEN ANTAGONIST POTENCY AND RECEPTOR BINDING

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1 The apparent dissociation constants (K_d) of four competitive antagonists of oxytocin were estimated from their ability to compete with [^{3}H]-oxytocin for binding sites in particulate fractions from rat uterine homogenates.

2 These apparent K_d values were not significantly different from the K_d values calculated from the published potency of each compound as an antagonist of oxytocin-induced uterine contractions.

3 These results support the conclusion that the binding sites for oxytocin are part of the receptor complex. Furthermore, 'spare receptors' for oxytocin do not appear to be present in significant quantities, and the relative potency of each antagonist appears to depend upon its affinity for the receptor site rather than its intrinsic activity.

4 The antagonists used in these studies were [*N*-acetyl, 2-*O*-methyltyrosine]oxytocin, [1-(β-mercapto-β,β-diethylpropionic acid)]oxytocin, [1-(β-mercapto-β,β-pentamethylenepropionic acid)]-oxytocin, and [1-(deaminopenicillamine), 4-threonine]oxytocin.

Introduction

Oxytocin-stimulated uterine contractions can be inhibited competitively by several synthetic analogues of oxytocin. These antagonists are either partial agonists, eliciting uterine contractions below the maximum response produced by oxytocin, or devoid of oxytocic activity. It seems apparent that the antagonists compete with oxytocin for uterine receptor sites. Antagonist activity has been explained by a low intrinsic activity (Ariëns, van Rossum & Simonis, 1956) or efficacy (Stephenson, 1956), or by a slow rate of dissociation of the partial agonist from the receptor site (Paton, 1961).

In view of the demonstration of specific binding sites for [^{3}H]-oxytocin in the uterus of several species (Soloff, Swartz, Morrison & Saffran, 1973; Soloff & Swartz, 1974; Soloff, Swartz & Steinberg, 1974; Soloff, 1975a) it is possible to estimate the binding affinities of the oxytocin antagonists directly and to correlate the values with antagonist activity. Four of the most potent oxytocin antagonists have been examined in the present studies.

Methods

Binding assay

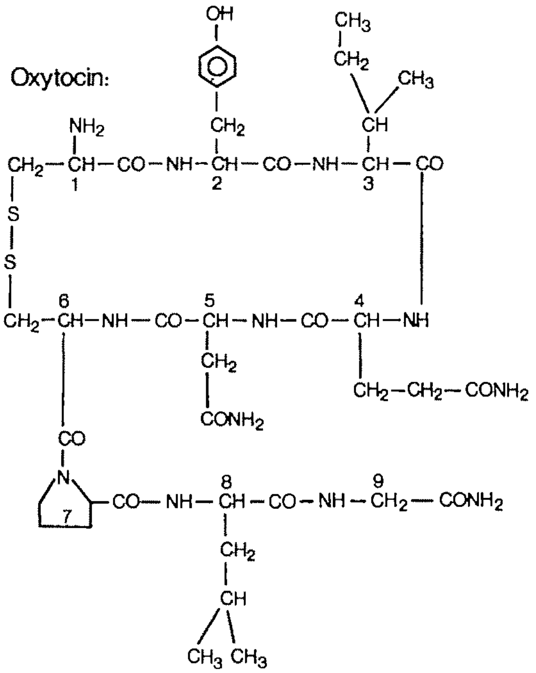
Uteri were removed from rats (CFE, Carworth, 175–200 g) which were injected subcutaneously with 5 µg of diethylstilbestrol dipropionate in 0.2 ml cottonseed oil on each of the two days before they were killed. The particulate fraction sedimenting between 1,000 *g* for 10 min and 165,000 *g* for 30 min was prepared and assayed for [^{3}H]-oxytocin binding activity (Soloff, 1975a).

Peptides

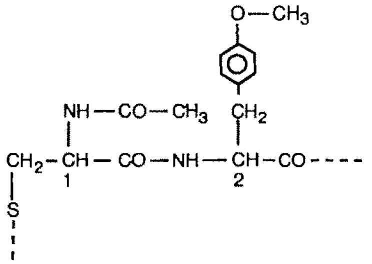
[Tyrosyl-^{3}H]-oxytocin, 31 Ci per mmol (Morgat, Hung, Cardinaud, Fromageot, Bockaert, Imbert & Morel, 1970) was synthesized by Schwarz-Mann and was reported to have full biological activity (452 iu per mg) in the rat isolated uterus assay (Stürmer, 1968; Fitzpatrick & Bently, 1968). More than 90% of the radioactivity migrated with authentic oxytocin upon thin layer chromatography (Soloff & Swartz, 1973).

Synthetic oxytocin (Syntocinon) was a gift from Sandoz, Ltd., Basel. [*N*-acetyl, 2-*O*-methyltyrosine]oxytocin was a gift from Drs K. Jošt and J.H. Cort, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague. [1-(β-Mercapto-β,β-diethylpropionic acid)]-oxytocin and [1-(β-mercapto-β,β-pentamethylene-propionic acid)]oxytocin were gifts from Dr V. du Vigneaud, Department of Chemistry, Cornell University. [1-(Deaminopenicillamine), 4-threonine]oxytocin was a gift from Dr M. Manning of this Department. [3-Proline]oxytocin was a gift from

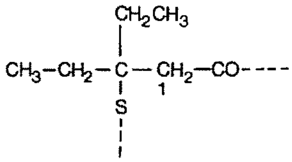
Oxytocin:



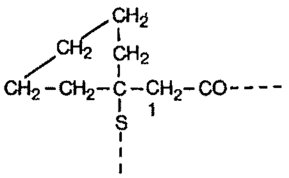
[*N*-acetyl, 2-*O*-methyltyrosine] oxytocin:



[1-(β-Mercapto-β,β-diethylpropionic acid)] oxytocin:



[1-(β-Mercapto-β,β-pentamethylenepropionic acid)] oxytocin:



[1-(Deaminopenicillamine), 4-threonine] oxytocin:

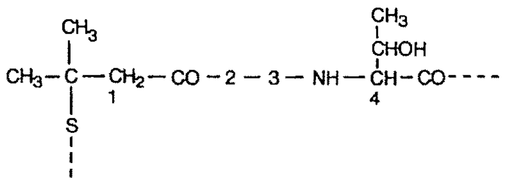


Figure 1 Structure of oxytocin and antagonists, with numbers indicating the position of the individual amino acid residues.

Dr R. Walter, Department of Physiology, University of Illinois Medical Center. Kallidin (lysyl-bradykinin) was purchased from Schwarz-Mann.

The structure of oxytocin and the antagonists [*N*-acetyl-2-*O*-methyl-tyrosine]oxytocin, [1-(β-mercapto-β,β-diethylpropionic acid)]oxytocin, [1-(β-mercapto-β,β, pentamethylenepropionic acid)]oxytocin and [1-(deaminopenicillamine), 4-threonine]oxytocin are shown in Figure 1.

Results

The amount of [^{3}H]-oxytocin bound to uterine particles was reduced in proportion to the log concentration of increasing amounts of nonradioactive oxytocin and the oxytocin antagonists (Figure 2). The regressions were parallel, indicating a common set of binding sites for the peptides. Kallidin, a potent uterotonic peptide which is structurally unrelated to oxytocin (Stewart, 1972), did not compete with [^{3}H]-oxytocin for binding sites at a molar ratio of greater than 1200 (Figure 2). [3-Proline]oxytocin, a virtually inert analogue of oxytocin (Roy, Havran, Schwartz & Walter, 1975), also did not compete for oxytocin binding sites in the dose range studied (Figure 2).

The regressions were analyzed as parallel line assays according to Finney (1964). The relative inhibitory potencies and 95% confidence limits of the antagonists, determined in 2 separate experiments for each, are shown in Table 1. Assuming that the analogues act by competition with oxytocin at a

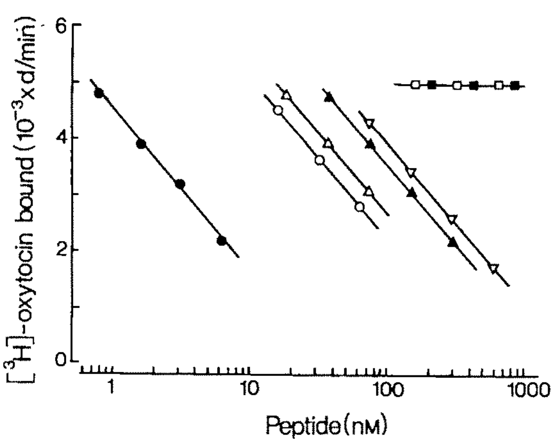


Figure 2 The binding of oxytocin and oxytocin antagonists to uterine particles. Each tube contained about 1 mg of particulate protein, 12,000 d/min of $[^3H]$-oxytocin (about 175 pg), and increasing amounts of nonradioactive peptide in 250 µl of Tris buffer (50 mM, pH 7.6, containing 5 mM $MgCl_2$ and 0.1% gelatin). Incubation was carried out for 1 h at 22°C and terminated by centrifugation of the tubes at 48,000 *g* for 30 minutes. The pellet was combusted to yield ^3H_2O. Each point is the mean of triplicates. Oxytocin (●); [1-(deaminopenicillamine), 4-threonine]oxytocin, (○); [1-(β-mercapto-β,β-pentamethylenepropionic acid]oxytocin, (△); [1-(β-mercapto-β,β-diethylpropionic acid]oxytocin, (▲); [*N*-acetyl, 2-*O*-methyltyrosine]oxytocin, (▽); [3-proline]oxytocin, (□); kallidin (■).

common binding site, the relative inhibitory potency for each compound provides a measure of its relative binding affinity. The relative inhibitory potency of each compound with respect to oxytocin was calculated from the results shown in Figure 2.

The apparent K_d for oxytocin binding, estimated from the linear segment of Scatchard (1949) plots was 1.6 ± 0.04 (s.e. mean) nM in 5 separate experiments (see Figure 3 for a representative plot). This value is comparable to the apparent K_d of 1.8 nM which was found in previous studies (Soloff & Swartz, 1974). The apparent K_d values calculated for each antagonist are given in Table 1.

The published activity of each antagonist (Table 1) is expressed as pA_2, the negative log of the molar concentration of antagonist which reduces the effect of a double dose of oxytocin to that of a single dose (Schild, 1947). The pA_2 value is equal to the negative log of the K_d for the antagonist-receptor interaction, assuming that one antagonist molecule binds to one receptor molecule (Ariëns & van Rossum, 1957). In all cases, the 95% confidence limits for the apparent K_d of each antagonist overlapped the K_d estimated from pA_2 (Table 1).

Discussion

Studies on oxytocin binding sites in the reproductive tract have been carried out on broken cell preparations (Soloff & Swartz, 1974; Soloff, *et al.*, 1974; Soloff, 1975a; Soloff, 1975b) in which there are no known biochemical correlates of oxytocin-receptor interaction. A causal relationship between the binding of oxytocin and the initiation of the contractile response is implicit, however, because several agonists have been shown to bind to uterine particles in approximate proportion to their uterotonic activities (Soloff & Swartz, 1974). The present studies show

Table 1 Comparison of the K_d values derived from antagonist activity and binding assay

	Bioassay		*Binding assay*	
Antagonist	*pA_2*	K_d*(10^{-8}M)*	*Relative inhibitory potency (%)*	K_d *(10^{-8}M)**
[*N*-acetyl, 2-*O*-methyltyrosine] oxytocin	7.03†	9.33	1.6 (1.3→2.0)⁺ 1.5 (1.3→1.8)	10 (12 →8.0)⁺ 11 (12 →8.9)
[1-(β-Mercapto-β,β-diethylpropionic acid)]oxytocin	7.24‡	5.75	2.5 (1.5→3.7) 2.5 (1.8→3.3)	6.4 (11 →4.3) 6.4 (8.9→4.8)
[1-(β-Mercapto-β,β-pentamethylene-propionic acid)]oxytocin	7.43§	3.71	4.2 (3.6→5.3) 3.4 (2.6→4.5)	3.8 (4.4→3.0) 4.7 (6.2→3.6)
[1-(Deaminopenicillamine), 4-threonine]oxytocin	~7.7‖	~2.0	6.1 (4.2→8.2) 5.9 (4.7→7.1)	2.6 (3.8→2.0) 2.7 (3.4→2.2)

* K_d oxytocin = 1.6 ± 0.04 nM. ⁺95% confidence limits.

† Krejčí, Kupková, Barth & Jošt (1973); ‡ Vavrek, Ferger, Allen, Rich, Blomquist & Du Vigneaud (1972); § Nestor, Ferger & Du Vigneaud (1975); ‖ Manning, Lowbridge & Sawyer (1975).

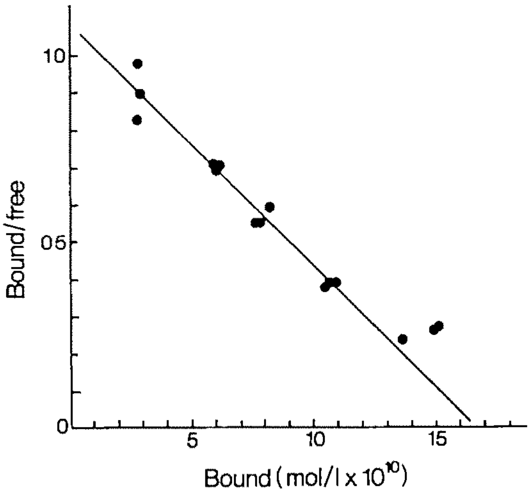


Figure 3 Scatchard analysis of oxytocin binding to uterine particles.

that the binding of the oxytocin antagonists as well was proportional to their potency as inhibitors of oxytocin-induced contractions by the isolated uterus. [3-Proline]oxytocin, a relatively inert analogue of oxytocin (Roy *et al.*, 1975) and kallidin, a potent uterotonic peptide (Stewart, 1972), but structurally unrelated to oxytocin, did not have any demonstrable affinity for oxytocin binding sites. These results, therefore, clearly indicate that the binding sites are components of the oxytocin receptor. The assumption that the same number of sites is available to oxytocin and the antagonists appears to be valid.

In addition to being an oxytocin antagonist, [*N*-acetyl, 2-*O*-methyl-tyrosine]oxytocin is a partial agonist (Krejči, Kupková, Barth & Jošt, 1973). The diethyl- and pentamethylene-propionic acid antagonists, however, appear to be devoid of oxytocic activity (Vavrek, Ferger, Allen, Rich, Blomquist & Du Vigneaud, 1972; Nestor, Ferger & Du Vigneaud, 1975). Partial agonists/competitive antagonists are postulated to be capable of full receptor occupancy, but they either dissociate very slowly from the receptor (Paton, 1961) or they lack some of the structural characteristics necessary for the initiation of the stimulus (Ariëns *et al.*, 1956; Stephenson, 1956). In the present studies the affinity of oxytocin receptors for the analogues has been estimated directly. The concurrence of the binding and antagonistic activities of the analogues demonstrates that the potency of each antagonist was a function of its affinity for the receptor site rather than its intrinsic activity.

A number of target cells appear to possess 'spare receptors' for drugs. For example, the guinea-pig ileum can be induced to contract maximally by drugs presumably when only a small proportion of the receptor sites are occupied (Stephenson, 1956; Nickerson, 1956; Ariëns, van Rossum & Koopman, 1960). Similar results were obtained with vascular smooth muscle (Furchgott, 1964). The concept of 'spare receptors' has received additional support from experiments in which the concentration of hormone giving a half-maximal response was found to be substantially less than the concentration of radioactive hormone binding to half of the receptor sites, the apparent K_d. Thus, ^{125}I-labelled insulin stimulated half-maximal lipogenesis from glucose in fat cells when about 2% of the receptors were occupied (Gammeltoft & Gliemann, 1973). Comparable results were found with human chorionic gonadotropin-stimulated production of cyclic adenosine 3,5′-monophosphate (AMP) and testosterone by rat testes (Catt & Dufau, 1973), glucagon-stimulated adenylate cyclase activity in rat liver plasma membranes (Birnbaumer & Pohl, 1973), glucagon-stimulated adenylate cyclase in a solubilized preparation from cat heart (Levey, Fletcher, Klein, Ruiz & Schenk, 1974), and ACTH-stimulated cyclic AMP production in adrenal cortical extracts (Lefkowitz, Roth, Pricer & Pastan, 1970). In contrast to these results, the present experiments indicate that there were no detectable spare receptors for the oxytocin antagonists because the apparent K_d of each antagonist estimated by the inhibition of oxytocin-induced uterine contractions was indistinguishable from the apparent K_d estimated by the ability to compete with [^{3}H]-oxytocin for uterine binding sites. Similar findings were reported for the binding of opiates to receptor sites in the guinea-pig intestine (Creese & Snyder, 1975).

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EFFECT OF PROSTAGLANDIN E_1 ON NEUROMUSCULAR TRANSMISSION IN THE RAT

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1 The effect of prostaglandin E_1 on neuromuscular transmission in the phrenic nerve-diaphragm muscle preparation of the rat was studied with intra- and extracellular recording techniques.

2 Prostaglandin E_1, in concentrations from 10 nM, induced intermittent failures in the generation of the end-plate potential in response to repeated indirect stimulation.

3 Failures appeared abruptly, the end-plate potential behaving in an all-or-nothing fashion. The effect occurred only at 36–38°C when the nerve was stimulated at 30–80 Hz and was reversible upon washing with drug-free solution.

4 Since miniature end-plate potentials were not affected, such failures must be attributed to a presynaptic action of prostaglandin E_1.

5 Extracellular recording suggested that prostaglandin E_1 prevented the action potential from reaching the nerve terminal.

Introduction

The modulation, by prostaglandins, of the release of noradrenaline from adrenergic nerve endings during stimulation of sympathetic nerves is well documented (Hedqvist, 1970b; Hedqvist, Stjärne & Wennmalm, 1970; Wennmalm, 1971). As regards cholinergic nerves, however, the role of prostaglandins in the release of acetylcholine is still controversial. Stimulation of the rat phrenic nerve-diaphragm muscle preparation (Ramwell, Shaw & Kucharski, 1965; Laity, 1969), of the cholinergic nerves to the rat stomach (Coceani, Pace-Asciak, Volta & Wolfe, 1967) or of the rabbit heart vagal nerves (Junstad & Wennmalm, 1974) liberates substantial amounts of prostaglandins. The results of several studies suggest moreover that prostaglandins may influence cholinergic neurotransmission (see Hedqvist, 1973). Some reports indicate that prostaglandins facilitate, others that they inhibit the release of acetylcholine from cholinergic nerve endings (Wennmalm & Hedqvist, 1971; Hahn & Patil, 1972; Ehrenpreis, Greenberg & Belman, 1973; Heilbronn, 1973; Hahn & Patil, 1974). Still other reports suggest, by showing that prostaglandins do not affect the release of acetylcholine, that prostaglandins do not act as modulators of neurotransmission in the parasympathetic neuroeffector system (Ginsborg & Hirst, 1971; Hadházy, Illés & Knoll, 1973; Marco & Coceani, 1973; Botting & Salzmann, 1974; Illés, Vizi & Knoll, 1974). To tackle this problem at a cellular level, the effects of prostaglandin E_1 on cholinergic neurotransmission were studied *in vitro* by intra- and extracellular recording techniques in the rat phrenic nerve-diaphragm muscle preparation. A preliminary report of this work has already appeared (Jansson, Hyvärinen, Tolppanen & Gripenberg, 1974).

Methods

Animals and preparations

The experiments were carried out *in vitro* with isolated left diaphragm muscles from adult male rats (180–220 g) of the Sprague–Dawley strain. The animals were anaesthetized with ether and killed, and the diaphragm muscle with the attached nerve was removed and immersed in a bathing solution. The muscle was stretched about 10% beyond its resting length and attached with stainless steel needles to a paraffin-lined Perspex plate that had a lens in the centre. The phrenic nerve was kept in mineral oil in a separate chamber. To avoid muscle twitches upon stimulation of the nerve, the ends of the muscle fibres were cut transversely (Barstad, 1962; Hubbard & Wilson, 1973).

Recording details

The experiments were begun 1 to 1.5 h after the muscle fibres had been cut. By that time the resting membrane potential (RMP) of the muscle fibres had decreased to a level at which an action potential could no longer be generated. Only surface fibres were studied. Intracellular recordings were made with glass microelectrodes drawn with internal glass fibres (Tasaki, Tsukakava, Ito, Wayner & Yu, 1968) and filled with 3 M KCl (resistance 5–20 MΩ). Most extracellular recordings were carried out with glass-insulated platinum microelectrodes (Wilska, 1940; Wolbarsht, MacNichol & Wagner, 1960). In some experiments, extracellular recordings were made with 3M NaCl-filled glass microelectrodes that had a resistance of less than 3 MΩ.

The phrenic nerve was stimulated with a bipolar platinum electrode. Pulses were of 0.04 ms duration and 2 to 3 times threshold strength. Microelectrodes were considered to be located in the end-plate region when end-plate potentials (e.p.ps) or miniature end-plate potentials (m.e.p.ps) were recorded that had a rise time of 0.8 ms or less. A criterion for the appropriate extracellular position of the microelectrode was the simultaneous recording of the arrival of the action potential at the nerve ending and the resultant end-plate current (e.p.c.). In the present paper, the abbreviation e.p.c. thus refers to an extracellular current and not to a true transmembrane current. Potentials were preamplified (using in intracellular recordings a d.c.-preamplifier with capacitance neutralization and an input resistance of 10^{12} Ω or, in extracellular recordings, a Tektronix 122 a.c.-preamplifier), displayed on an oscilloscope and photographed or recorded on FM-tape. M.e.p.p. amplitudes were measured from the screen of a storage oscilloscope after FM-recorded material had been replayed.

Solutions

The composition of the bathing solution was as follows (mM): NaCl 136.0, KCl 5.6, $MgCl_2$ 1.3, $CaCl_2$ 2.2, PO_4^{3-} 1.3, $NaHCO_3$ 16.0 and glucose 11.0. Because 5 mM KCl has been shown to induce nerve block in cut preparations (Randić & Straughan, 1964), the potassium concentration was reduced to 2.8 mM when e.p.ps or e.p.cs were studied. The solution was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 which resulted in a pH of 7.3 at room temperature. A peristaltic pump infused the solution into the bathing chamber at a constant rate of 3–6 ml min^{-1}. The temperature of the bathing medium was recorded with a thermistor. Prostaglandin E_1 was added to the bathing medium from a 10 mM stock solution in 95% ethanol.

Results

Effect of prostaglandin E_1 on resting membrane potential of muscle fibres and on spontaneous m.e.p.ps

The effects of prostaglandin E_1 on the RMP and spontaneous m.e.p.ps were studied in uncut preparations that had been bathed in solutions containing 5.6 mM KCl. Prostaglandin E_1 at 0.1 μM had no definite effects on either of these parameters. The RMP of muscle fibres in control preparations maintained at 37°C was 76.7 ± 5.8 mV, whereas during exposure to prostaglandin E_1, the RMP was 76.7 ± 7.6 mV (means ± s.d. of 82 and 87 muscle fibres, respectively, in 12 preparations). The mean amplitude of m.e.p.ps after at least 10 min of treatment with 0.1 μM prostaglandin E_1 was 0.54 ± 0.26 mV, as compared with 0.52 ± 0.23 mV under control conditions (mean ± s.d.). Corresponding m.e.p.p. frequencies were 9.60 ± 5.24 and 7.37 ± 5.20 m.e.p.ps s^{-1}, respectively. Prostaglandin E_1 did not affect the distribution of m.e.p.p. amplitudes in a way indicative of a clear-cut biological effect (Figure 1).

Neuromuscular transmission in the cut diaphragm muscle preparation

One hour after the muscle fibres had been cut, with the preparation having been maintained at room temperature, the RMP of the muscle fibres had decreased to about −35 mV. During the following 3 to 4 h, there was a further gradual depolarization. The RMP of muscle fibres during this period was −31.6 ± 10.9 mV (mean ± s.d. of 116 fibres in 31 preparations). At such a low RMP, no action potential was generated, but an e.p.p. 1–10 mV in amplitude was elicited in response to nerve stimulation. When a tetanic stimulation was applied to the nerve, the amplitude of the first e.p.ps decreased. In cut preparations, unlike those treated with tubocurarine, the amplitude of the e.p.p. remained at a conveniently recordable level after this initial run-down (see also Lilleheil, 1965; Hubbard & Wilson, 1973).

Failures in the e.p.p. response

As the frequency of the indirect stimulation was progressively increased, the e.p.p. sooner or later failed intermittently. The stimulation frequency at which such failures occurred varied considerably among different junctions. In 12 preparations, 56% of all end-plates tested (86 fibres) at 37°C showed no failures when stimulated for a total of 100 s, the stimulation frequency being increased from 10 to 100 Hz in 10 Hz steps every tenth second. The remaining 38 cells failed somewhere between 20 and 90 Hz. When failures started to appear, the e.p.p. did not decrease in amplitude but rather responses to some stimuli in the

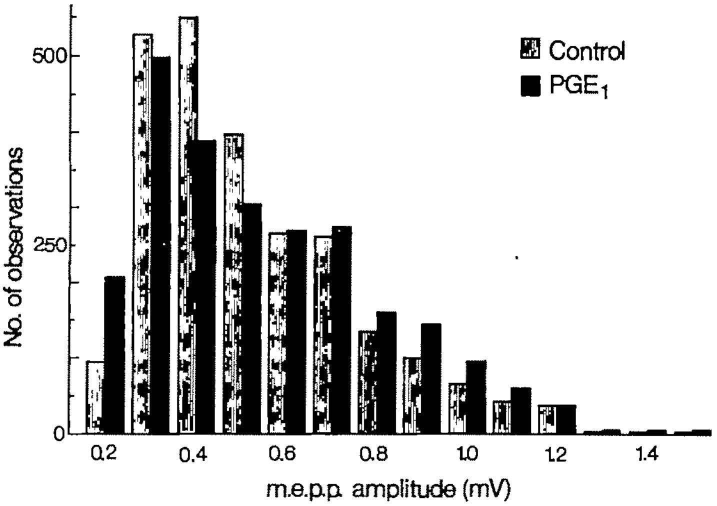

Figure 1 Lack of effect of prostaglandin E$_1$ (PGE$_1$) 0.1 µM on m.e.p.p. amplitude in the rat diaphragm muscle preparation *in vitro*. A total of 12 preparations were used. In eight of these, recordings were first made from seven control cells after which prostaglandin E$_1$ was infused into the bathing chamber. Recordings were begun 10 min after the addition of prostaglandin E$_1$. In the other four preparations, recordings were first made in prostaglandin E$_1$ and control recordings were begun after 15–30 min of washing with drug-free solution. All experiments were carried out at 37°C. A total of 2496 and 2453 m.e.p.ps were analyzed during control (lined columns) and prostaglandin E$_1$-conditions (solid columns) respectively.

train disappeared altogether, the e.p.p. behaving like an all-or-nothing phenomenon. When the stimulation frequency was decreased from, for example, 50 to 5 Hz, failures disappeared. If the stimulation frequency was resumed or increased, a total block of the e.p.p. occurred. This kind of failure has previously been described by Krnjević & Miledi (1958; 1959) as presynaptic failure of neuromuscular propagation and results from an impaired conduction of the action potential in the terminal branch of motor nerve fibres.

Prostaglandin E$_1$-induced failures in the e.p.p. response

When the effects of prostaglandin E$_1$ were being studied, only such cells were examined which did not exhibit spontaneous failures in the e.p.p. response to tetanic stimulation. Prostaglandin E$_1$, concentrations from 10 nM, induced intermittent failures in the generation of the e.p.p. within a few minutes when the nerve was stimulated at 30–80 Hz at 37°C. As a rule, continuing high-frequency stimulation after prostaglandin E$_1$-induced failures had appeared, resulted in a complete block of the e.p.p. As was also found to be true for spontaneous failures, prostaglandin E$_1$-induced failures occurred abruptly. Only occasionally did the e.p.p. (or e.p.c.) amplitude definitely decrease before failures occurred. The amplitude of single e.p.ps appearing after failures was as a rule greater than average. Further, intermittent prostaglandin E$_1$-induced failures, like spontaneous failures, were abolished by decreasing the stimulation frequency. Blockade of the e.p.p. was often preceded by a prolongation of the latency between the stimulation artefact and the beginning of the e.p.p.

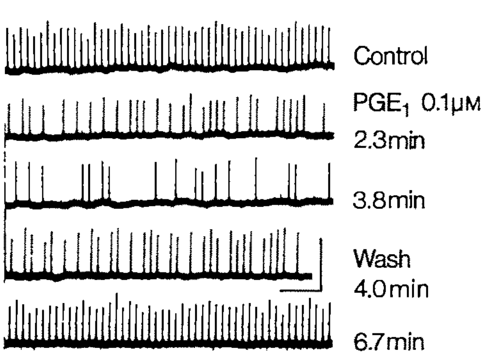

Figure 2 Effect of prostaglandin E$_1$(PGE$_1$) 0.1 µM on the generation of e.p.ps in the cut phrenic nerve-diaphragm muscle preparation of the rat in response to continuous indirect stimulation at 80 Hz *in vitro*. All records are from the same cell. The temperature was maintained at 36–37°C and the RMP varied between −30 and −28 mV throughout the experiment. Calibrations: vertical, 3 mV; horizontal, 0.1 s.

The results of a typical experiment are illustrated in Figure 2. The nerve was continuously stimulated at

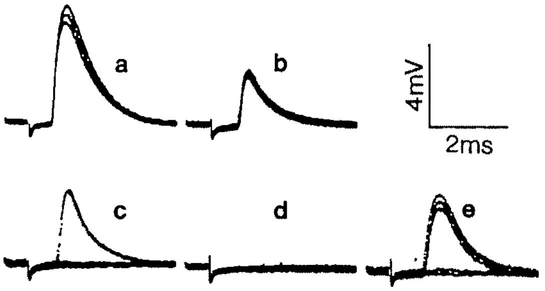

Figure 3 Effect of the stimulation frequency on the generation of e.p.ps in the cut phrenic nerve-diaphragm preparation of the rat treated with prostaglandin E_1 0.1 μM *in vitro.* The temperature was maintained at 37–38°C and the RMP varied between −40 and −34 mV except in (e), in which the RMP was −24 mV. The nerve was initially stimulated at 30 Hz (a) after which the stimulation frequency was gradually increased in 5–10 Hz steps. After 1 h of continuous stimulation at increasing frequencies, the stimulation frequency reached 80 Hz (b) whereupon the frequency was increased to 100 Hz (c) and a few minutes later to 105 Hz (d). Record (e) was made 16 min after washing with prostaglandin E_1-free solution was begun, the stimulation frequency being maintained unchanged at 105 Hz. Each frame is composed of 3–10 superimposed traces.

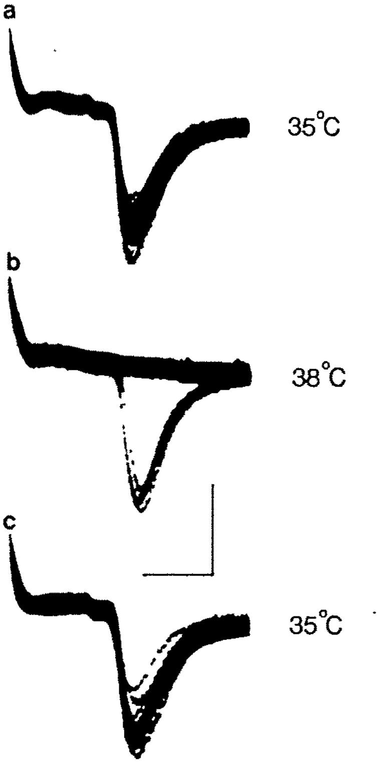

Figure 4 Temperature-dependence of the prostaglandin E_1-induced block of the terminal nerve spike and subsequent e.p.c. failure. Records were obtained extracellularly from a cut phrenic nerve-diaphragm muscle preparation of the rat *in vitro.* Prostaglandin E_1 was used in a concentration of 1 μM throughout the experiment. The stimulation frequency was 70 Hz. In (a) the temperature was 35°C and the preparation had been exposed to prostaglandin E_1 for 19.5 min; (b) was made 1 min after the temperature had been raised to 38°C. Note that although the e.p.c. fails in the majority of traces, a few stimulations have resulted in the generation of an e.p.c. while the corresponding nerve spikes are obscured by the baseline; (c) was obtained less than 1 min after the temperature had been readjusted to 35°C. Each frame is composed of about 35 superimposed traces. Calibrations vertical, 0.3 mV; horizontal, 1 ms.

80 Hz, and during control conditions every stimulation resulted in the generation of an e.p.p. While stimulation of the nerve was continued, prostaglandin E_1 was infused into the bathing chamber at 0.1 μM and within 2 to 3 min the e.p.p. suddenly and intermittently failed. Upon washing with drug-free solution, the number of failures gradually decreased until the e.p.p. response was completely normal. In cells where the e.p.p. was totally blocked, washing with drug-free solution resulted in a sudden appearance of the full-size e.p.p. In one single experiment, the reappearing e.p.p. gradually increased in size until normal amplitude was achieved.

Two experiments were carried out with uncut, curarized preparations and altogether 7 experiments were conducted with preparations treated with high concentrations of Mg^{2+} to block muscle contractions. Extensive experiments with curarized preparations were hampered by the pronounced tetanic run-down of the e.p.p. during these conditions. In Mg^{2+}-treated preparations, positive effects with prostaglandin E_1 were recorded. It seemed, however, that high concentrations of Mg^{2+} opposed the prostaglandin E_1-effect. Instead of appearing within 2 to 3 min as in a cut preparation, prostaglandin E_1-induced failures often did not appear until after about 10 minutes. It is not clear if this is a specific Mg^{2+}-effect or whether the cut preparation is in some way more susceptible to the effects of prostaglandin E_1.

Effect of the concentration of prostaglandin E_1

The effect of prostaglandin E_1 remained the same when used in a wide range of concentrations. The same abrupt intermittent failure in the tetanic e.p.p.

response and subsequent block of the e.p.p. occurred within a few minutes of treatment with prostaglandin E_1 in a concentration ranging from 10 nM to 10 μM. Concentrations lower than 10 nM were not tested. The possibility of an inverse correlation between the concentration of prostaglandin E_1 and the stimulation frequency required to obtain a positive prostaglandin E_1-effect, was not examined.

Dependence on stimulation frequency

At no concentration tested did prostaglandin E_1 induce failures or transmission blockade when the nerve was stimulated below 10 Hz. There appeared to be a threshold frequency below which, at a particular junction, no prostaglandin E_1-effects could be obtained. In one-fifth of all trials in prostaglandin E_1, e.p.p. failures were recorded at a stimulation frequency of 15 Hz. Upon stimulation at 30 or 50 Hz, 45% and 60%, respectively, of all end-plates showed a constant failure or block of the e.p.p. which was reversed upon washing with drug-free solution. At higher stimulation frequencies, the incidence of conduction failures in control solution was high (cf. page 388), making the study of the prostaglandin E_1-effect difficult. In the experiment illustrated in Figure 3, there was no failure upon stimulation at 30 Hz despite exposure to prostaglandin E_1 at 1 μM. When the stimulation frequency was progressively increased, failures and, ultimately, a block of the e.p.p. occurred at 100 and 105 Hz, respectively.

Temperature dependence

The prostaglandin E_1 effect proved extremely temperature-dependent. The effect was readily obtained at 37–38°C but was not seen at temperatures below 35°C. As may be seen in Figure 4, there was no failure in the generation of the extracellularly recorded e.p.c., despite exposure to 1 μM prostaglandin E_1, as long as the temperature was maintained at 35°C. When the temperature was raised, the e.p.c. started to fail intermittently within 1 min after the temperature had reached 38°C. Readjusting the temperature to 35°C immediately restored the response. In this particular experiment, prostaglandin E_1 was washed out at this stage of treatment. The preparation could now be maintained at 38°C for 15 min while a normal e.p.c. was recorded from the same cell that had previously failed at this temperature when exposed to prostaglandin E_1.

Effect of prostaglandin E_1 on the nerve terminal spike and end-plate current

Currents generated by action potentials in the presynaptic nerve terminal and by the action of concomitantly-released transmitter on the postsynaptic receptor can be recorded simultaneously with extracellular techniques (Katz & Miledi, 1965a,b). The results of the present m.e.p.p. experiments and the mode of failure of the e.p.p. favour a presynaptic site of action for prostaglandin E_1 in inhibiting the stimulus-evoked release of acetylcholine. Extracellular recordings were made from the end-plate to see if the e.p.p. failures were due to a failure of invasion of the nerve terminal by the nerve spike or whether the impulse invaded the terminal but failed to release transmitter.

With extracellular techniques, positive effects with prostaglandin E_1 were obtained at seven junctions in seven preparations. The generation of the extra-

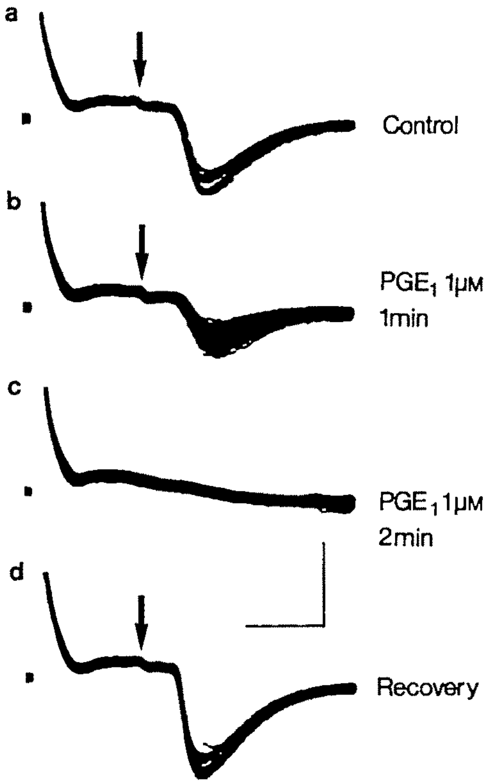

Figure 5 Effect of prostaglandin E_1 (PGE$_1$) 1 μM on the extracellularly-recorded terminal nerve spike and the e.p.c. in the cut phrenic nerve-diaphragm muscle preparation of the rat *in vitro*. The stimulation frequency was 60 Hz and the preparation was maintained at 38°C. The terminal nerve spike is recorded as a small deflection in the baseline (arrow) preceding the e.p.c. In (c) the blockade of the e.p.c. is preceded by a block of the terminal nerve spike. Each frame is composed of about 30 superimposed traces. Calibrations: vertical, 0.2 mV; horizontal, 1 ms.

cellularly recorded e.p.c. was blocked by prostaglandin E_1 (0.1 μM and 1 μM) in the same way as was the generation of the e.p.p. The e.p.c. failed abruptly although in one cell the e.p.c. amplitude decreased gradually before failures occurred. In six out of seven successful experiments, a failure in the e.p.c. response was consistently preceded by a block of the action current. In the seventh experiment, a summation of potentials originating from different junctions was apparently recorded which resulted in a terminal spike persisting even when the e.p.c. was blocked by prostaglandin E_1. A typical experiment is illustrated in Figure 5. Under control conditions, the action potential arriving at the nerve terminal is recorded as a small deflection preceding the e.p.c. Within 1.5 min after the introduction of prostaglandin E_1, the amplitude of the e.p.c. was decreased but the nerve spike continued to invade the nerve terminal. After an additional 1.5 min the terminal spike as well as the e.p.c. were blocked. One minute of washing with prostaglandin E_1-free solution restored both the spike and the e.p.c.

Discussion

The present results confirm and extend preliminary observations (Jansson *et al.*, 1974) showing that prostaglandin E_1 depresses release of acetylcholine evoked by nerve impulses. Under these conditions, the e.p.p. or e.p.c. abruptly begins to fail intermittently in an all-or-nothing manner. As judged from present extracellular recordings, this probably results from a partial or complete failure of the nerve impulse to invade the nerve terminal. The presence of m.e.p.ps of unchanged amplitude excludes the possibility of a direct effect on the postsynaptic membrane.

The simplest explanation for our observations is that prostaglandin E_1 depolarizes the presynaptic nerve fibre membrane (see Sjöstrand, 1972) thereby causing a presynaptic inhibition of acetylcholine release (Eccles, Schmidt & Willis, 1962). However, the unchanged m.e.p.p. frequency as well as the all-or-nothing character of the e.p.p. block after treatment with prostaglandin E_1 indicate that the unmyelinated nerve terminal was not affected by the hypothetical membrane depolarization (see Miledi & Slater, 1966). Instead the block is apparently located proximally to the nerve terminal, perhaps at one of the last nodes of Ranvier (Katz & Miledi, 1968) or at the vulnerable branching of peripheral nerve ramifications (Krnjević & Miledi, 1958; 1959). As judged from extracellular recordings (Hedqvist, 1970a; Jansson *et al.*, 1974), conduction in the nerve trunk is not affected by prostaglandins.

The absolute refractory period of fast mammalian nerve fibres is about 0.5 ms. This is compatible with a stimulation frequency of 2000 Hz, which is possible for only short periods (Gasser & Grundfest, 1936; Lowitzsch & Hopf, 1972). Spikes elicited even at millisecond intervals do progressively level off. But as pointed out by Krnjević & Miledi (1959), it is surprising that conduction failure appears at a stimulation frequency well below 100 Hz. The mechanisms involved in spontaneous failure of the e.p.p. have been discussed at some length by Krnjević & Miledi (1959) who suggested that the deleterious effect of prolonged nerve stimulation may be focused on the terminal nerve branches which, while running within the diaphragm muscle, '. . . are fully exposed to the effects of substances released by active muscles'. Our results raise the possibility that spontaneous failure of the e.p.p. might be mediated by prostaglandins released from either the muscle or the nerve. This possibility is supported by the demonstrated release of prostaglandins upon stimulation of a rat phrenic nerve-diaphragm muscle preparation (Ramwell *et al.*, 1965; Laity, 1969). On the other hand, prostaglandin E_1 was effective only during certain critical conditions (temperature 36–38°C, tetanic stimulation rate). In other words, e.p.p.-failures induced by exogenous prostaglandin E_1 occurred only when the safety factor for impulse conduction was brought close to one (see Krnjević & Miledi, 1958; 1959). The marginal nature of the prostaglandin E_1-effect could be taken as an indication against a profound physiological role of prostaglandins in neuromuscular transmission.

In conclusion, exogenous prostaglandin E_1 induces, upon repetitive indirect stimulation, intermittent failures and ultimate block of the e.p.p. in the cut phrenic nerve-diaphragm muscle preparation of the rat. This effect is reversible and it is achieved by preventing the invasion of the nerve terminal by the action potential. A protective role of prostaglandins in neuromuscular transmission might be considered. Only from the results of further studies will a fuller understanding of these processes become available.

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THE USE OF DIFFERENT AGONISTS IN ANTAGONIST AFFINITY CONSTANT ESTIMATIONS

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1 The affinities of 4 muscarinic antagonists were estimated on intact pieces of guinea-pig ileum using the agonists carbachol and pentyl trimethylammonium both in separate experiments and in the same experiment.

2 The apparent affinities were slightly but consistently higher when estimated from the responses produced by pentyl trimethylammonium than when estimated from the responses produced by carbachol.

3 This difference was greatly reduced or abolished if totally denervated logitudinal muscle strips were used rather than intact pieces of ileum. It is therefore suggested that the difference is due to the presence of receptors in the ganglionic layer.

4 To explain the difference in apparent affinity of the antagonists these receptors can not be identical to the muscarinic receptors on the smooth muscle.

5 In addition they can not be nicotinic ganglionic receptors as the difference did not appear to be affected by the presence or absence of hexamethonium.

Introduction

Abramson, Barlow, Mustafa & Stephenson (1969) compared the affinity constants of a number of muscarinic antagonists on guinea-pig ileum using different agonists. The largest difference they observed was for the one comparison made between carbachol and pentyl trimethylammonium (pentyl TMA) but the difference was not statistically significant. However real differences in mean log affinity as large as 0.1 log units might not have been evident in their experiments. The possibility that there is a real, albeit small, difference in apparent affinity has now been investigated.

Methods

Intact pieces of guinea-pig ileum or logitudinal muscle strips were prepared as described by Edinburgh Staff (1968) and Paton & Rang (1965) respectively. Strips were only considered to be totally denervated if they failed to respond to the ganglion stimulant, pentyl TMA ($NH_2 . C_6H_4 . CH_2 . CH_2 . N^+Me_3I^-$; Barlow & Franks, 1971). Such strips could only be obtained from animals weighing between 500 and 600 g. Otherwise animals weighing between 150 and 400 g were used. The extent to which Auerbach's plexus remained attached was estimated after staining with methylene blue.

The preparations were suspended in an organ bath containing Tyrode solution at 36°C and through which air was bubbled. The Tyrode solution was of the following composition (mM): Na^+ 149.2, K^+ 2.7, Mg^{2+} 1.1, Ca^{2+} 1.8, Cl^- 143.2, HCO_3^- 11.9, $H_2PO_4^-$ 0.4, SO_4^{2-} 1.1, glucose 5.6 and hexamethonium bromide 0.276 (unless specified otherwise). The responses produced by carbachol or pentyl TMA were recorded isotonically with a differential transformer. When intact pieces of ileum were used a weight of between 0.5 and 0.8 g was used to load the lever; with muscle strips, 0.2 to 0.6 g was used. Responses were produced throughout each experiment every 90 s, the agonist was removed after 17 s and a second wash applied after another 30 seconds. The apparatus was described by Abramson *et al.* (1969).

The affinity constants of the antagonists were measured using either carbachol or pentyl TMA as described by Edinburgh Staff (1968): Responses were produced by two concentrations of agonist, one double the other, used alternately. The high-low response sequence was maintained in the presence of the antagonist with adjusted concentrations of agonist. Affinity constant estimations were also made using both agonists in the same experiment. In these a high-low response sequence was maintained using the four possible combinations of the four agonist solutions in an order determined by a Latin square.

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The drugs used were kindly provided by R.B. Barlow except where specified otherwise: benziloyl tropine methyliodide (BTrMe), carbaminoylcholine chloride (carbachol) (BDH Chemicals), diphenylacetoxyethyldimethylethyl ammonium iodide (desoxyl-achesine), diphenylhydroxyacetoxyethyldimethylethylammonium bromide (lachesine), hexamethonium bromide (Koch-Light), *n*-pentyl triethylammonium iodide (pentyl TEA), *n*-pentyl trimethylammonium iodide (pentyl TMA).

Results

The affinity constants K_{aff} of 3 antagonists were estimated using pentyl TMA and carbachol in

Table 1 Mean values of log affinity constant (Log K_{aff}) obtained for 3 muscarinic antagonists using intact pieces of guinea-pig ileum. Estimates were made from responses to carbachol or pentyl TMA, the agonists being used in separate experiments

Antagonist	*Range of Antagonist Concentrations M*	*Mean Log K_{aff} ± S.E.M.* Carbachol	Pentyl TMA
BTrME	6–200 × 10^{-10}	10.129 ± 0.044 (27)	
	10–39 × 10^{-10}		10.206 ± 0.068 (9)
Lachesine	0.5–2 × 10^{-8}	8.817 ± 0.027 (26)	8.963 ± 0.011 (5)
Pentyl TEA	0.3–8 × 10^{-4}	4.558 ± 0.016 (24)	4.593 ± 0.029 (5)

(*n*) = number of estimations made. Benziloyl tropine methyliodide (BTrMe), *n*-pentyl triethylammonium iodide (pentyl TEA).
According to Student's *t*-test or Mann–Whitney U test, the probability of observing these differences is > 0.2 in each case if the null hypothesis were true.

Table 2 Values of log affinity constant (Log K_{aff}) estimated from responses of intact pieces of guinea-pig ileum to carbachol and pentyl TMA, both agonists being used in each experiment

Antagonist	*Antagonist Concentration M*	*Log K_{aff}* Carbachol	*Pentyl TMA*	*Difference*
BTrMe	10 × 10^{-10}	10.000	10.079	+0.079
		9.778	9.963	+0.125
		10.146	10.230	+0.084
Pentyl TEA	2.5 × 10^{-4}	4.611	4.667	+0.056
	5 × 10^{-4}	4.588	4.806	+0.218
	15 × 10^{-4}	4.444	4.597	+0.153
Desoxylachesine	13 × 10^{-7}	7.378	7.549	+0.171
		7.509	7.671	+0.162
		7.549	7.618	+0.069
		7.549	7.567	+0.018
	26 × 10^{-7}	7.477	7.732	+0.255
		7.544	7.576	+0.032
		7.642	7.823	+0.181
		7.681	7.699	+0.017
	40 × 10^{-7}	7.735	7.844	+0.109
		7.691	7.884	+0.203
		7.688	7.833	+0.145
		7.639	7.803	+0.164
	50 × 10^{-7}	7.834	7.946	+0.112
		7.491	7.742	+0.251
		7.604	7.721	+0.117
		7.534	7.835	+0.301

Benziloyl tropine methyliodide (BTrMe), *n*-pentyl triethylammonium iodide (pentyl TEA).
According to the sign test the probability of observing 22 differences all in the same direction is < 0.0001 if the null hypothesis were true.

Table 3 Values of log affinity constant (Log K_{aff}) of desoxylachesine (5×10^{-7}M) estimated on intact pieces of ileum from the dose-ratios in the absence and then in the presence of hexamethonium (2.76×10^{-4}M)

No Hexamethonium			*Hexamethonium*		
Log K_{aff}			*Log K_{aff}*		
Carbachol	*Pentyl TMA*	*Difference*	*Carbachol*	*Pentyl TMA*	*Difference*
7.292	7.881	+0.589	7.193	7.790	+0.597
7.589	7.719	+0.130	7.362	7.688	+0.326
7.584	7.619	+0.035	7.428	7.511	+0.083
7.668	7.740	+0.070	7.571	7.619	+0.048
		mean +0.206			mean +0.263

separate experiments (Table 1), the antagonists being chosen to cover as wide a range of potency as possible. As found by Abramson *et al.* (1969), although the mean value of K_{aff} obtained using pentyl TMA is for each antagonist higher than that obtained using carbachol, the difference is too small to be statistically significant.

Affinity constants were therefore estimated using both agonists in the same experiment (Table 2). In each of 22 experiments the affinity constant estimated from the pentyl TMA responses was higher than that calculated from the carbachol responses. This difference is statistically significant, $P < 0.0001$ by sign test. Desoxylachesine was used instead of lachesine in these experiments because it is in the same potency range as lachesine but its onset and offset of action is appreciably faster.

As hexamethonium had been present in all the experiments covered by Tables 1–2, its removal might have been expected to affect the difference in the affinity constants if the difference were due to nicotinic receptors in the ganglionic layer. The apparent affinity of desoxylachesine was therefore measured in the absence of hexamethonium and with hexamethonium present throughout in its normal concentration of 2.76×10^{-4}M (Table 3). The difference in apparent

Table 4 The dose-ratio produced by hexamethonium (2.76×10^{-4}M) on intact pieces of ileum using carbachol and pentyl TMA in the same experiment

Dose-ratio		
Carbachol	*Pentyl TMA*	*Difference*
1.20	1.30	+0.10
1.43	1.02	−0.41
1.22	1.07	−0.15
1.02	1.09	+0.07
		mean −0.10

Table 5 The effect of partial or total denervation on the difference in the log of the affinity constant (Log K_{aff}) when estimated from responses to carbachol and from responses to pentyl TMA, both agonists being used in the same experiment. (a) Longitudinal muscle strips with less than 30% of the area covered by the nerve plexus. (b) Totally denervated longitudinal muscle strips

Antagonist	*Antagonist Concentration M*	*Log K_{aff}*		
		Carbachol	*Pentyl TMA*	*Difference*
(a) Desoxylachesine	50×10^{-7}	7.563	7.631	+0.068
		7.428	7.486	+0.058
		7.567	7.609	+0.042
		7.458	7.560	+0.102
				mean +0.068
(b) Pentyl TEA	2.5×10^{-4}	4.526	4.530	+0.004
		4.651	4.653	+0.002
	15×10^{-4}	4.456	4.473	+0.017
		4.621	4.596	−0.025
		4.444	4.444	+0.000
				mean −0.004

Comparable figures with intact pieces of ileum are given in Table 2. The Mann–Whitney U test was used to test whether the groups were drawn from the same population. The probabilities were 0.018 ($n_1 = 3$, $n_2 = 5$) and 0.014 ($n_1 = n_2 = 4$) for pentyl TEA and desoxylachesine if the null hypothesis were true.

affinity did not appear to be affected by the absence of hexamethonium. The dose-ratio produced by this concentration of hexamethonium itself was also measured using carbachol and pentyl TMA in the same experiment (Table 4). The dose-ratio estimated from the pentyl TMA responses were not consistently different from those estimated from the carbachol responses. It is therefore unlikely that the difference in apparent affinity can be explained in terms of nicotinic ganglionic receptors.

If the difference in apparent affinity were due to non-nicotinic receptors in the ganglia, the difference would not be observed in totally denervated muscle strips. Using such preparations the difference in apparent affinity was found to be greatly reduced or abolished (Table 5). It therefore seems likely that the difference in apparent affinity of an antagonist when estimated from responses to carbachol and pentyl TMA can be attributed to the presence of Auerbach's plexus.

Discussion

By using two agonists in the same experiment it has been possible to show that there is a real but small difference in the apparent affinity of an antagonist when measured using carbachol as compared with pentyl TMA. As this difference was greatly reduced or abolished when denervated preparations were used and was not affected by hexamethonium it must be due to the presence of non-nicotinic receptors in Auerbach's plexus although the exact site and mode of action can not be established from these experiments. These ganglionic receptors may also account for the small difference in affinity noted by Barlow & Tubby (1974) when using the agonists 3,3-dimethylbutylacetate and carbachol and that observed by Furchgott & Bursztyn (1967) and Waud (1969) when measuring the affinities of partial agonists using different methods.

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THE PHARMACOLOGY OF ANAPHYLAXIS IN THE CHICKEN INTESTINE

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1 The Schultz-Dale phenomenon has been demonstrated in several circular smooth muscle strips of oesophagus, crop, duodenum, jejunum and ileum taken from young and adult domestic fowl sensitized actively to crystalline bovine albumin or horse plasma.

2 The ileal strips contract to acetylcholine, histamine, 5-hydroxytryptamine (5-HT), prostaglandins E_1, E_2, $F_{2\alpha}$, bradykinin and bovine slow reacting substance of anaphylaxis (SRS-A). Marked seasonal and individual variations in the responsiveness of gut tissues to these exogenous agonists were noted.

3 Antagonism of contractions to histamine by mepyramine suggests the existence of H_1-histamine receptors in chicken ileum. Blockade of 5-HT-induced contractions by methysergide shows the preponderance of 'D'-musculotropic tryptamine receptors.

4 Failure of selective receptor antagonists of acetylcholine, histamine and 5-HT to modify the Schultz-Dale reaction suggests the nonparticipation of aminergic mechanisms in this reaction.

5 Partial to complete blockade of the Schultz-Dale reaction by a prostaglandin receptor antagonist (polyphloretin phosphate, PPP); prostaglandin synthetase inhibitors (sodium meclofenamate and phenylbutazone); inhibitors of synthesis and release of histamine and SRS-A (PR-D-92-EA, M&B 22948, diethylcarbamazine citrate, and PPP) and an inhibitor of proteinases (aprotinin) strongly suggests the involvement of vasoactive lipids and polypeptides in the anaphylactic response of chicken ileum to specific antigen.

Introduction

In 1910, Schultz reported contraction of the isolated intestinal strip from a sensitized guinea-pig on exposure to the specific antigen. Three years later, Dale (1913) demonstrated a similar response of the uterus. Since then, antigen-induced smooth muscle contraction *in vitro* has become a standard technique for demonstrating anaphylactic hypersensitivity. Subsequently this 'Schultz-Dale' effect has been described in the oesophagus (Califano & Scapagnini, 1968) and ileum (Dale, 1965; Dale & Okpako, 1969; Okpako, 1970) of guinea-pig; jejunum (Aitken, Deline & Eyre, 1975), pulmonary (Eyre, 1971; Aitken *et al.*, 1975), mesenteric and hepatic (Holroyde & Eyre, 1975) blood vessels of calf. However, Hirata & Cambell (1961) failed to demonstrate a Schultz-Dale reaction in the intestine of passively sensitized adult domestic fowl.

Forceful fluid defaecation (suggestive of increased gastrointestinal motility and secretions) during acute anaphylaxis in the chicken has been documented repeatedly (Lecomte & Beaumariage, 1958; Pedersoli, 1973; Chand & Eyre, unpublished). This prompted us to study the Schultz-Dale phenomenon in several parts of the gastrointestinal tract of actively sensitized chickens. In order to illustrate the possible nature of the principal chemical mediator(s) participating in anaphylaxis in chicken intestine, known specific antagonists, together with non-steroidal anti-inflammatory agents and some new anti-anaphylactic drugs were included in the study.

Methods

Seventy-nine adult and ten one week old chickens (White or Brown Leghorn) were used. The birds were sensitized either to bovine albumin (ICN Pharmaceuticals, Cleveland, Ohio) (80 mg kg^{-1}, i.v.) (Makinodan, Wolfe, Goodman & Ruth, 1952) or horse plasma (1 ml kg^{-1}, i.v.). On the seventh day of sensitization, overnight-fasted chickens were killed by intravenous injection of pentobarbitone. Sections of intestine were immediately removed and transferred to cold aerated Krebs-Henseleit solution (Everett, 1966; Everett & Mann, 1967). Segments (approximately 2 cm) of duodenum, jejunum and ileum were removed and their contents washed out with Krebs solution. Each segment of intestine was cut spirally and the resultant single strip then bisected longitudinally to produce two identical strips (containing predominantly circular smooth muscle). This technique allowed comparisons of responses of 'twin' strips to exogenous agonists and antigen in the presence and absence of antagonists; one strip of each pair always serving as a control (untreated by antagonist) in each experiment (Eyre, 1971). A transverse crop strip was carefully dissected without stretching the tissue (Everett, 1966). An oesophageal strip was prepared by longitudinally cutting a 2 to 4 mm wide segment of oesphagus. Each tissue was suspended in a 30 ml isolated organ bath (Phipps & Bird, Richmond, Va.) containing Krebs solution at 37°C, gassed with 5% CO_2 in O_2 mixture and was allowed to equilibrate for at least 1 h under a resting tension of 3 grams.

Responses of the strips were recorded with an E & M isotonic myograph transducer (Narco Instruments, Houston, Tex.) connected to an E & M (Narco) Desk Model, 4-Channel Physiograph (DMP-4B) pen recorder. All the strips were exposed to each dose of agonist for 1 to 5 min followed by 5 to 15 min recovery period. Three-point dose-response curves to each agonist were established. It was noted that both strips usually produced approximately equal responses to agonists. However, any pairs which exhibited marked differences in sensitivity or showed excessive spontaneous activity were discarded.

After establishing dose-response curves to randomly injected agonists, a concentration of each agonist producing approximately equivalent reproducible responses (about 50% maximum) was chosen. A predetermined concentration of an antagonist was added to one of the tissues and left in contact for 30 minutes. Each agonist was then randomly retested in both tissues as before to establish new dose-response curves. The effectiveness and specificity of the antagonist was determined by measuring the 'dose-ratio' i.e. the ratio of the doses of agonist which give equal responses in the presence and absence of antagonist (Gaddum, Hameed, Hathway & Stephens, 1955). Both the strips were 'challenged' with crystalline bovine albumin (25 $\mu g\ ml^{-1}$) or horse plasma (0.3 ml in 30 ml) to produce a Schultz-Dale reaction. The Schultz-Dale contraction of each muscle strip was measured and the degree of inhibition caused by the antagonist was expressed as a percentage reduction of the unantagonized response.

Drugs

Histamine diphosphate, bradykinin triacetate, 5-hydroxytryptamine (serotonin) creatinine sulphate and atropine sulphate were purchased from Nutritional Biochemical Corp., Cleveland, Ohio; acetylcholine chloride from Sigma Chemical Co., St. Louis, Mo.; phenylbutazone sodium (Butazone) from Stevenson, Turner and Boyce, London, Ontario. Slow-reacting substance of anaphylaxis (SRS-A) was obtained from calf lung and assayed on guinea-pig ileum (Burka & Eyre, 1975).

The following drugs were obtained as gifts: mepyramine maleate from Poulenc Ltd., Montreal, Quebec; methysergide bimaleate from Sandoz, Basle, Switzerland; diethylcarbamazine citrate (Franocid, DECC) from Burroughs Wellcome Ltd., London, England; sodium meclofenamate from Parke, Davis and Co., Detroit, Mich.; prostaglandins E_1, E_2 and $F_{2\alpha}$ from Upjohn Co., Kalamazoo, Mich.; polyphloretin phosphate (PPP, Leo 101K) from Leo, Helsingborg, Sweden; 2-9-propoxyphenyl-8-azapurin-6-one (M&B 22948) from May and Baker Ltd.; FPL 55712 from Fison Limited; aprotinin (Trasylol) from Boehringer Ingelheim (Canada) Ltd., Montreal, Quebec; 5,5-dimethyl-11-oxo-5H,11H-(2)-benzopyrano(4,3-g) (1)benzopyran-9-carboxylic acid ethanolamine salt (PR-D-92-EA) from Pharma Research, Pointe-Claire, Quebec. All the drugs were dissolved in Krebs solution and the concentrations expressed as salts. FPL 55712 was dissolved initially in distilled water and diluted further in Krebs solution.

Results

Threshold doses of exogenous agonists and antigens on several intestinal strips are presented in Table 1. Histamine (1 nM to 0.1 μM) sometimes produced a slight relaxation but as the doses of histamine were increased, only contractile responses were recorded. In general, acetylcholine (ACh) was 100 to 1000 times more active than histamine and 5-hydroxytryptamine (5-HT) on most tissues. It was noted that tissues were measurably more sensitive to agonists in the spring and summer compared with the winter. Crop strips in summer were extremely sensitive to 5-HT. Responses to histamine and 5-HT were slow in onset and recovery (1 to 5 min) depending upon the dose used and sensitivity of the tissues.

Threshold doses and effects of bradykinin, prostaglandins and bovine SRS-A were variable. However, in summer months, it was noted that prostaglandins E_1, E_2 (0.1 to 10 $ng\ ml^{-1}$) and $F_{2\alpha}$ (1 to 100 $ng\ ml^{-1}$) exhibited strong consistent dose-dependent contractions of ileal strips ($n=6$), whereas in winter, sensitivity to prostaglandins was so low that frequently it was not possible to elicit any contractile response ($n=5$).

The Schultz-Dale reaction was characterized by a contraction associated with increased 'spontaneous' activity after 1 to 5 min of antigen exposure to the tissues. The absolute intensity of contraction and spontaneity was highly variable depending upon the organ used; the sensitizing and challenging dose of antigen, type of antigen (bovine albumin or horse plasma), and season. The tissue reaction was highly consistent between 'twin' strips taken from the same organ on the same occasion.

A number of crystallized albumins and globulins (dog, bovine, pig, rabbit, sheep, goat) (1 to 100 µg ml^{-1}) and horse or bovine plasma (0.01 to 0.3 ml in 30 ml) were devoid of any visible effect on intestinal strips from five nonsensitized chickens.

Intestinal strips (2/6) from chickens sensitized to bovine albumin exhibited cross (nonspecific) Schultz-Dale reaction to horse and pig albumins but not to globulins. However, tissues ($n=6$) from birds sensitized to horse plasma did not show any cross reaction to albumins and globulins of other species. Furthermore, a few gut strips (7/36) from bovine albumin-sensitized birds did not contract to challenge with bovine albumin. Alimentary tissues from one week old chicks were about 100 to 1000 times more sensitive to exogenous agonists but the *in vitro* anaphylactic responses were similar to those of adult chickens.

Ileal strips ($n=4$) passively sensitized to chicken anti-horse sera (diluted with aerated Krebs solution, 1:10, at 4°C for 2 h) also showed strong Schultz-Dale contractions to horse plasma. The nonsensitized second strip of each pair did not respond to antigen challenge.

Antagonists

The inhibitory activity of the specific antagonists on ACh, histamine, 5-HT and the Schultz-Dale anaphylactic response are shown in Table 2.

Atropine At lower concentrations (0.05 and 0.5 µg ml^{-1}), atropine selectively antagonized ACh. At higher doses (1 to 10 µg ml^{-1}) atropine also inhibited histamine (dose-ratio 5 to 60) and 5-HT (dose-ratio 10 to 100) without modifying the Schultz-Dale reaction in any way (not included in Table 2).

Mepyramine (0.5 to 1.0 µl mg^{-1}) specifically inhibited histamine without inhibiting the Schultz-Dale effect. Larger doses (2 to 10 µg ml^{-1}) of mepyramine partially contracted the intestinal strips and inhibited 5-HT (dose-ratio 15 to 50), also reducing the Schultz-Dale response an average 40% ($n=3$) (not included in the Table). In some (3/7) ileal strips, mepyramine reversed the histamine-induced contraction (Figure 1a) into a relaxant effect.

Methysergide (0.2 and 2.0 µg ml^{-1}) selectively antagonized 5-HT without modifying the responses to histamine, ACh or antigen.

In some additional experiments ($n=5$) (not included in the Table), combined addition of atropine, mepyramine and methysergide (1 µg ml^{-1} of each) also failed to alter the anaphylactic Schultz-Dale reaction. Furthermore, tachyphylaxis to 5-HT induced by repeated injection of 5-HT ($n=3$) also failed to inhibit the Schultz-Dale phenomenon.

Sodium meclofenamate (1 to 10 µg ml^{-1}) did not inhibit contractions to ACh, histamine, 5-HT and antigen. Larger doses of meclofenamate (20 and 50 µg ml^{-1}) exhibited varying degrees of nonspecific blockade of histamine, ACh, 5-HT and strongly inhibited the Schultz-Dale anaphylactic response (60 and 85% block).

Phenylbutazone (10 to 500 µg ml^{-1}) antagonized ACh, histamine and 5-HT, but inhibited the Schultz-Dale response only at the highest dose (500 µg ml^{-1}).

Diethylcarbamazine citrate (DECC) (10 to 100 µg ml^{-1}) inhibited exogenous agonists slightly (dose-ratio 2 to 10) (not included in the Table). However, large doses strongly antagonized histamine, ACh, 5-HT and Schultz-Dale contraction. DECC itself contracted gut strips and in three preparations, it also reversed the SRS-A-induced contractions.

Table 1 Threshold dose ranges of some spasmogens on certain circular smooth muscle strips of gastro-intestinal tract of adult domestic fowl

	Agonists (M)				*Antigens*	
Source of the tissues	*5-HT*	*Histamine*	*Actyl-choline*	*Carbachol*	*BA* µg ml^{-1}	*HP* *ml/30 ml*
Anterior and posterior oesophagus	10^{-8} to 10^{-8}	10^{-7} to 10^{-6}	10^{-9} to 10^{-7}	10^{-10} to 10^{-8}	0.1 to 2	0.01 to 0.02
Crop	10^{-10} to 10^{-8}	10^{-7} to 10^{-6}	10^{-9} to 10^{-8}	10^{-10} to 10^{-9}	0.1 to 2	0.01 to 0.02
Small intestine (duodenum, jejunum, ileum)	10^{-7} to 10^{-4}	10^{-7} to 10^{-4}	10^{-9} to 10^{-7}	10^{-10} to 10^{-8}	1 to 10	0.02 to 1
Caecum	—	10^{-7} to 10^{-6}	10^{-10} to 10^{-9}	—	—	—

BA=bovine albumin; HP=horse plasma.

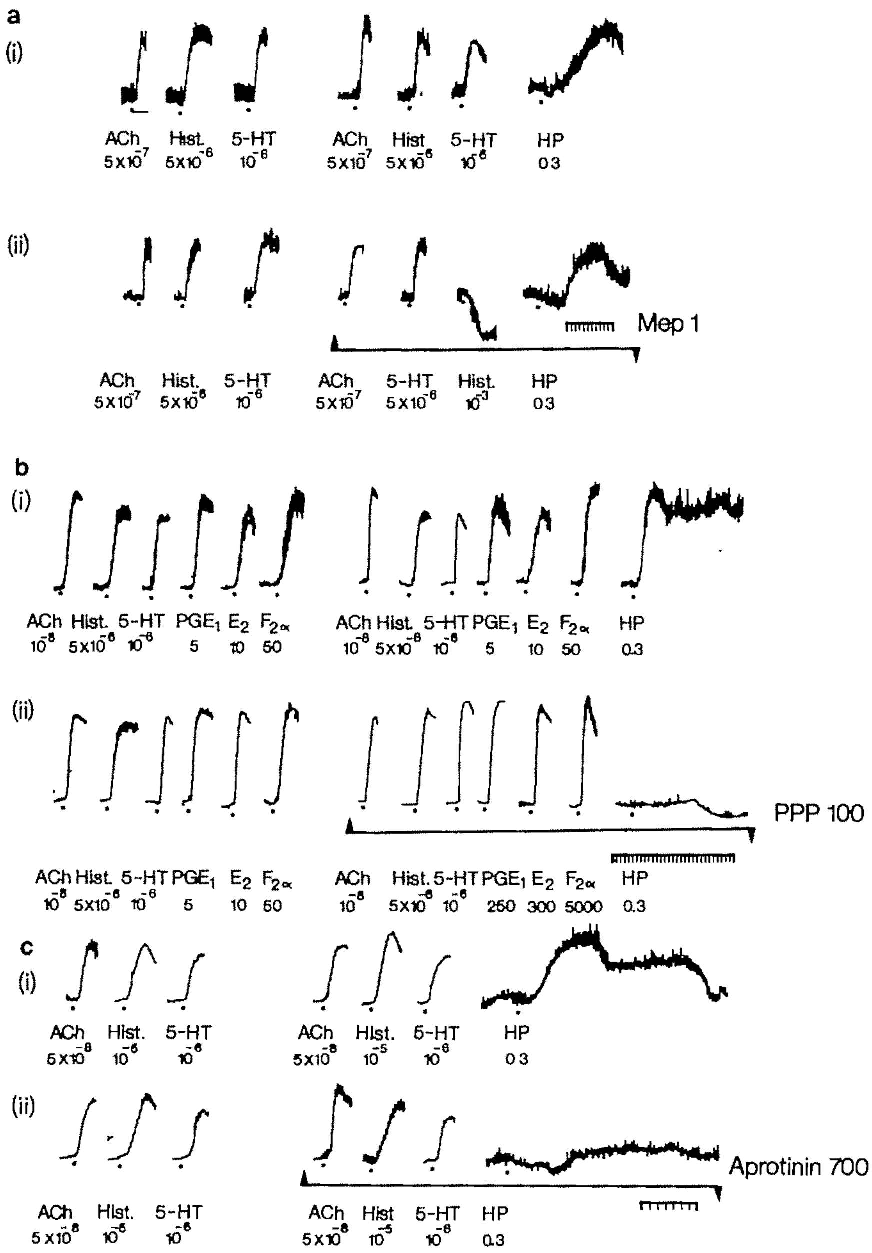

Figure 1 Three pairs (a), (b) and (c) of isolated spiral strips of ileum taken from three adult domestic fowl sensitized to horse plasma (1 ml kg^{-1}, i.v.). All strips contracted to acetylcholine (ACh), histamine (Hist), 5-hydroxytryptamine (5-HT) and horse plasma (HP) in 30 ml Krebs-Henseleit solution gassed with 95% O$_2$ and 5% CO$_2$ at 37°C. Both strips of the second pair (b) also showed contractions to prostaglandins (PG E$_1$, E$_2$ and F$_{2\alpha}$). In the second (ii) strip of each pair an antagonist was present in the bath fluid between the arrows shown: (a, ii) mepyramine maleate (Mep) reversed histamine contraction, reduced 5-HT-induced response but did not inhibit ACh and Schultz-Dale reaction. (b, ii) Polyphloretin phosphate (PPP) inhibited responses to prostaglandins E$_1$, E$_2$ and F$_{2\alpha}$ (ng ml^{-1}) and Schultz-Dale response. (c, iii) Aprotinin (KIU ml^{-1}) did not affect responses to histamine, ACh and 5-HT but blocked Schultz-Dale phenomenon. Doses of agonists (histamine, ACh, 5-HT) are in Molar concentration; antagonists in μg ml^{-1} and horse plasma in ml. Time marker indicates minutes.

Polyphloretin phosphate (PPP) (100 μg ml^{-1}) reduced or blocked the anaphylactic response and reversed or inhibited contractions to prostaglandins E_1, E_2, and $F_{2\alpha}$ ($n=4$) (Figure 1b) without antagonizing ACh, histamine or 5-HT.

FPL 55712 at all doses (0.1 to 100 μg ml^{-1}) did not inhibit contractions to histamine, ACh, 5-HT and antigen. However, addition of compound FPL (1 to 100 μg ml^{-1}) before or after antigen-challenge invariably inhibited antigen-induced spontaneous activity of the intestine, but did not block actual contractions.

PRD-92-EA (10 to 200 μg ml^{-1}) antagonized ACh, histamine, 5-HT slightly and reduced antigen-induced spontaneity only. Large doses strongly inhibited exogenous agonists and completely inhibited the Schultz-Dale anaphylactic responses.

M&B 22948 (10 to 60 μg ml^{-1}) did not possess any inhibitory activity towards histamine, ACh, 5-HT and antigen-induced contractions. At a higher dose (200 μg ml^{-1}), it had little or no inhibitory effect upon exogenous agonists, but blocked the Schultz-Dale response completely.

Table 2 Dose-ratios of histamine, 5-hydroxytryptamine (5-HT) and acetylcholine, and percent inhibition of Schultz-Dale contractions in chicken ileum *in vitro*, in the presence of antagonists

Antagonist	*μg ml^{-1}*	*Dose-ratios of agonists*			*% Inhibition of Schultz-Dale reaction (Mean ± s.d.)*
		Histamine	*5-HT*	*Acetylcholine*	
Atropine	0.05	1,1,1,1	1,1,1.5,1	300,900,500, 1000	0 (4)
	0.5	1,2,1.5,1	1,1,1,2.5	3050,6000, 6050,2000	0 (4)
Mepyramine	0.05	10,5,10	1,1,1	1,1,1	0 (3)
	0.1	30,120,80	1.5,1,1	1,1,1	0 (3)
	1.0	500,300,165, 250	2,1,5,2	1,2,1.5,1	0 (4)
Methysergide	0.2	1,2,1,1	20,15,10,4	1,1,2,1	0 (4)
	2.0	1,1,1,3	40,100,40,35	1,1,1,2	0 (4)
Sodium meclofenamate	1.0	1,1,1	1,1,1	1,1,1	0 (3)
	10.0	3,1,1,1	10,1,2,1	1,1,1,1	0 (4)
	20.0	5,2,1,1,1	10,30,2,5,2	5,2,1,2,1	60 ± 25 (5)
	50.0	5,20,1,2	25,10,30,10	5,2,2,5	85 ± 25 (4)
Phenylbutazone	10	1,2,1.5	1,2,1.5	2,1.5,2	0 (3)
	100	3,10,2.5,6,3,5	2,22,5,10,2	1,2,5,3,6,2.5	0 (6)
	500	6,5,10,60,5	5,30,2,10,6	5,20,6.5,100, 15	100 (5)
DECC	500	3,6,25	12,25,60	2,1,1	0 (3)
	4000	100,250,150 irreversible	300,250, irreversible,450	2,10,15,100	90 ± 15 (4)
PPP	100	1,1,1,1,1	1,1,1,1,1	1,1,1,1,1	60 ± 25 (5)
FPL 55712	0.1	1,1,1	1,1,1	1,1,1	0 (3)
	1.0	1,1,1	1,1,1	1,1,1	0 (3)
	10.0	1,1,1	1,1,1	1,1,1	0 (3)
	100.0	1,1,3	1,2,1	1,1,1	0 (3)
PRD-92-EA	200	1,3,2,1	1,10,2,1	3,2,1,10	0 (4)
	500	25,100,15,10	10,2,50,110	20,3,1.5,2	100 (4)
M & B 22948	30	1,1,1,1	1,1,1,1	1,1,1,1	0 (4)
	60	1,1,1	1,1,1.5	1,1,1	0 (3)
	200	2,1,1,1	2,1,1.5,2	1,2,1,3	100 (4)
Aprotinin	100 KIU ml^{-1}	1,1,1	1,1,1	1,1,1	0 (3)
	700 KIU ml^{-1}	1,1,1	1,1,1	1,1,1	75 ± 25 (4)
	1000 KIU ml^{-1}	1,1,1,1.5	1.5,1,1,2	1,1,1.5,1	80 ± 20 (4)

Aprotinin (700 to 1000 kallikrein inactivating units (KIU) ml^{-1}) blocked the Schultz-Dale response without interfering with the responses of histamine, 5-HT or ACh (Figure 1c).

Discussion

Many intestinal strips (oesophagus, crop, duodenum, jejunum and sometimes ileum) exhibited marked spontaneous activity which was resistant to atropine, methysergide and mepyramine. Earlier, Everett (1966) had reported similar observations which she ascribed to intrinsic myogenic activity. This spontaneity was inhibited by the addition of catecholamines (noradrenaline and isoprenaline) which also produced dose-related reduction in basic tone of tissues. Furthermore, reduction of spontaneous activity of these smooth muscle strips could be achieved by halving the amount of Ca^{2+} in the Krebs solution and by lowering the temperature (37°C to 25°C). This resulted in significant (50 to 75%) reduction in sensitivity to exogenous spasmogens and antigens. These findings are in general agreement with the fact that for the anaphylactic release of histamine (and other vasoactive substances like SRS-A) responsible for the antigen-induced contraction of smooth muscles, an appropriate concentration of Ca^{2+} ions and optimum temperature of tissue bathing fluid is essential (Mongar & Schild, 1957; Grosman & Diamant, 1974). It is generally assumed that calcium participates in intracellular reactions of the release process.

The seasonal and individual variability in the responsiveness of intestinal tissues to exogenous agonists, particularly insensitivity to histamine and 5-HT in winter, supports the similar earlier finding on the crop (Everett, 1966). The delay of 1 to 3 min in the initiation of contraction of intestinal strips of adult chickens to 5-HT and histamine may be due to the presence of dense connective tissue barriers retarding the diffusion of these drugs to sites of action (Everett, 1966). Similar reasons may explain the delay in recovery from contractions.

In spite of this individual and seasonal variability to exogenous agonists, the only marked difference in the character of the Schultz-Dale anaphylactic reactions was a longer latent period (2 to 10 min) in the initiation of contraction followed by greater spontaneity in winter compared to shorter latent period (1 to 5 min) and relative absence of increased spontaneity in summer. No obvious correlation could be established between the responsiveness of tissues to exogenous agonists and anaphylactic Schultz-Dale contraction.

Schultz-Dale anaphylactic responses were observed in the following decreasing order of intensity in the crop, oesophagus, jejunum, duodenum and ileum strips. The Schultz-Dale reaction could not be demonstrated in caecal strips. This variability in the intensity of this *in vitro* anaphylactic reaction may be attributed to differences in mast cell numbers (Wight, 1970; Carlson & Hacking, 1972), smooth muscle sensitivity, their avidity for cytotropic antibody, or their histamine (or other vasoactive spasmogen) contents. Demonstration of Schultz-Dale reactions in the intestine of actively sensitized chickens suggest the presence of tissue-fixed antibodies, capable of interacting with specific sensitizing antigens on challenge, resulting in the release of pharmacological vasoactive substances (most probably from mast cells) and thus producing contraction of smooth muscles. Reaginic antibodies mediating passive cutaneous anaphylaxis (PCA) in chickens have been found to belong to γ_1, a subclass of IgG immunoglobulins (Faith & Clem, 1973).

Desensitization of tissues to subsequent antigenic challenge was a common feature of this reaction. Desensitization to antigen(s) has been widely reported (Dale, 1965; Dale & Okpako, 1969; Okpako, 1970; Eyre, 1971).

It is pertinent to mention that antigen-induced contractions and increased spontaneity were maintained for 30 min to 2 h in spite of repeated washings in many preparations. This observation suggests continuous release of vasoactive substances from sensitized tissues once the immunological reaction has been triggered by specific antigen.

The cross (non-specific) Schultz-Dale reaction observed on some strips may be due to the existence of some common antigenic determinants on albumin molecules of different species (cow, horse and pig).

Reduction or blockade of histamine and 5-HT-induced responses by large doses of atropine may be due to its well known antihistamine (Schild, 1947; Chand & Eyre, 1976 and antitryptamine ('D' musculotropic) effects (Gaddum & Picarelli, 1957; Chand & Eyre, 1976). Lack of antianaphylactic effect of atropine suggests a lack of involvement of cholinergic mechanisms in intestinal anaphylaxis in chickens.

Slight relaxant effects to low doses of histamine on chicken small intestine may be attributed to catecholamine-releasing action from the intestine (Everett & Mann, 1967). However, the possibility of the presence of H_2-histamine receptors (Black, Duncan, Durant, Ganellin & Parsons, 1972) sensitive to low doses of histamine and mediating slight relaxant effects cannot be ruled out. Rather, this possibility is further substantiated by the observations that complete blockade of H_1-receptors (mediating contractile responses) by mepyramine (Ash & Schild, 1966) reversed the contractile responses to a relaxant effect. This may indicate an unmasking of H_2-receptor activity after complete H_1-blockade. The small contractile responses to larger doses of mepyramine may be due to its known histamine-releasing properties (Mota & DaSilva, 1960).

It has been reported that classical H_1-receptor antagonists inhibit anaphylaxis in domestic fowl (Lecomte & Beaumariage, 1958) but this has not been confirmed (Pedersoli, 1973). Furthermore, in the present study, mepyramine (a preferential H_1-histamine receptor antagonist) (Chand & Eyre, 1975) is ineffective in blocking antigen-induced contractions. This findings seems to support the earlier suggestion that in chicken anaphylaxis, histamine may be playing a minor role (Pedersoli, 1973).

5-HT antagonists have been reported to be ineffective in protecting chickens from anaphylactic shock (Lecomte & Beaumariage, 1958; Pedersoli, 1973). Resistance of the Schultz-Dale reactions to methysergide appears to strengthen this earlier view and 5-HT may not be an important mediator of anaphylaxis in domestic fowl (Lecomte & Beaumariage, 1958). Furthermore, intestinal strips (insensitive to higher doses of histamine and 5-HT) or tissue strips (rendered tachyphylactic to 5-HT by frequent injection of 5-HT) showed equal sensitivity to anaphylactic Schultz-Dale contraction. These observations, together with the ineffectiveness of atropine, mepyramine and methysergide in blocking Schultz-Dale contractions strongly suggest that cholinergic, histaminergic or tryptaminergic mechanisms are of low significance in this anaphylactic reaction.

Sodium meclofenamate and phenylbutazone are non-steroidal anti-inflammatory agents, possessing strong prostaglandin synthetase inhibitory activity (Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972). Sodium meclofenamate inhibits certain actions of kinin, SRS-A, prostaglandin $F_{2\alpha}$ and anaphylactic bronchoconstriction in the guinea-pig (Collier & Shorley, 1963; Berry & Collier, 1964; Collier & Sweatman, 1968). At low concentrations, neither antagonist had any inhibitory activity against exogenous agonists. These findings are in agreement with an earlier report (Burka & Eyre, 1974). At higher doses, both the drugs showed marked inhibitory activity against exogenous agonists and the Schultz-Dale reaction. Thus, the non-steroidal anti-inflammatory agents are nonspecific, possessing a variety of modes of action, both at the sites of the mediators synthesis and at receptor sites. The inhibition of the Schultz-Dale response by prostaglandin-synthetase inhibitors (meclofenamates and phenylbutazone) suggests a role for prostaglandins in the reaction. Specific inhibition of anaphylaxis by PPP further supports a role for prostaglandins.

DECC has been reported to release histamine (Deline, Eyre & Wells, 1973) which may account for the contractile responses observed. Subsequent blockade of histamine responses by DECC may be attributed to its known antihistaminic activity (Orange, Valentine & Austen, 1968). It has earlier been reported that DECC inhibits responses to histamine and 5-HT (Burka & Eyre, 1974) and the Schultz-Dale reaction (Eyre, 1971). High doses of DECC have been reported to inhibit immunological release of SRS-A (Orange *et al.*, 1968; Burka & Eyre, 1975) and the inhibition of the Schultz-Dale phenomenon by large doses of DECC appears to suggest the formation and release of SRS-A from intestinal tissues but lack of specificity of DECC on a variety of exogenous agonists makes such a conclusion tenuous. Furthermore, antigen-induced release of SRS-A and histamine from chicken lung and intestine *in vitro* could not be demonstrated (Chand & Eyre, unpublished).

Alimentary tissues of several species have the ability to synthesize, release and metabolize prostaglandins. The pharmacological actions (relaxation or contraction) vary greatly depending upon the species, strain, region of gastrointestinal tract, and type of smooth muscles (circular or longitudinal) involved. It has been suggested that generation of prostaglandins appears to maintain the tone of the gut (Wilson, 1974). In general prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ produce contraction of longitudinal muscles in several species, whereas circular smooth muscles are relaxed by prostaglandins E_1, E_2 and contracted by prostaglandin $F_{2\alpha}$ (Wilson, 1974). In contrast, prostaglandins E_1, E_2 and $F_{2\alpha}$ contract circular smooth strip of ileum of chicken. Thus it may be suggested that prostaglandins are involved in the maintenance of tone of circular smooth muscles of chicken gut. The inhibitory activity of PPP on Schultz-Dale reaction may be attributed to the inhibition of formation and release of SRS-A and histamine (Strandberg, 1973; Foucard & Strandberg, 1975; Burka & Eyre, 1975) as well as to the blockade of prostaglandin receptor sites *per se* (Eakins, Karim & Miller, 1970).

FPL 55712 has been introduced as a potent specific SRS-A receptor antagonist (Augstein, Farmer, Lee, Sheard & Tattersall, 1973). This compound does not inhibit Schultz-Dale contraction in chicken intestine (this paper) or in guinea-pig ileum (Eyre, unpublished). However, inhibition of antigen-induced spontaneity associated with Schultz-Dale contraction by FPL 55712 possibly suggests that released SRS-A may be playing a role in antigen-induced spontaneous activity but not in antigen-induced contractions.

PR-D-92-EA is a new antianaphylactic agent chemically related to disodium cromoglycate (Stewart, Devlin & Freter, 1974). The inhibition of the Schultz-Dale response by this agent may be attributed to the complex inhibitory effects on the immunological release of histamine (Stewart *et al.*, 1974), SRS-A (Burka & Eyre, 1975), degranulation of mast cells (Stewart, personal communication), and antagonistic activity towards bradykinin, 5-HT, prostaglandins E_2, $F_{2\alpha}$ and SRS-A (Possanza, Bauen & Stewart, 1974).

M&B 22948 is a derivative of methylxanthine and is another new inhibitor of reagin-mediated anaphylaxis (Broughton, Chaplen, Knowles, Lunt, Pain, Wooldridge, Ford, Marshall, Walker & Maxwell, 1974). The inhibition of the Schultz-Dale reaction by this compound may be due to the inhibition of formation and release of histamine and SRS-A (Broughton *et al.*, 1974; Burka & Eyre, 1975) by virtue of blocking cyclic 3′,5′-adenosine monophosphate phosphodiesterase.

Bradykinin is known to contract many intestinal smooth muscles, e.g. rat fundus strip, rabbit duodenum and large intestine and to relax rat duodenum and hen rectal caecum (Rocha e Silva, 1970). The significance of avian kinin (ornithokinins) in pathophysiological processes is not known (Eisen, 1971). The effect of bradykinin on chicken ileum was variable, i.e. no effect or slight to marked contractions at high doses. This needs further study. The reduction or blockade of Schultz-Dale anaphylactic reaction by aprotinin may be attributed to the inhibition of intestinal kallikreins (Zeitlin, 1971) and/or other intestinal proteases (Webster, 1970).

Prostaglandin-synthetase inhibitors (sodium meclofenamate, phenylbutazone, indomethacin), prostaglandin-receptor antagonist (PPP), inhibitors of formation and release of histamine and SRS-A (PPP, PR-D-92-EA, M&B 22948, DECC, disodium cromoglycate) and the kallikrein inhibitor aprotinin have been found to inhibit *in vivo* systemic anaphylaxis significantly. These agents are 100% effective in inhibiting the antigen-induced bradycardia and increased central venous pressor and are 50 to 90% effective in blocking the arterial hypotension in systemic anaphylaxis of chiken (Chand & Eyre, unpublished).

This study suggests that cholinergic, histaminergic and tryptaminergic mechanisms are of low importance in intestinal anaphylaxis of chickens. Results with non-steroidal anti-inflammatory drugs and the so-called anti-anaphylactic agents implicate the vasoactive lipid substances. More specifically since FPL 55712, a proposed SRS-A blocking agent (Augstein *et al.*, 1973) does not inhibit anaphylactic smooth muscle contraction, whereas PPP, a prostaglandin-receptor antagonist (Eakin *et al.*, 1970) strongly inhibits anaphylaxis; it may be tentatively suggested that prostaglandins are more important than SRS-A. On the other hand FPL 55712 and DECC both antagonized the increased 'spontaneity' of the alimentary tract during anaphylaxis, which might implicate SRS-A in the rhythmic low-amplitude movements, rather than in the contracture *per se*. It is pertinent to mention that bovine SRS-A exogenously applied to the chicken ileum, caused a small contraction but a large increase in spontaneous movement. The anti-anaphylactic action of aprotinin, a potent kallikrein inhibitor, also implicates kinins in intestinal Schultz-Dale reactions of chickens.

It would not necessarily be valid to extend these observations to explain systemic anaphylaxis of chickens in which many other organs participate and where the relative significance of chemical mediators may be quite different. It would be reasonable to suggest, however, that Schultz-Dale anaphylaxis may contribute to increased intestinal motility ('forced defaecation') which is a consistent feature of systemic anaphylaxis in chickens. It is a matter for speculation, but certainly a possibility, that local anaphylaxis may contribute to the aetiology of important diseases of the avian digestive tract.

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ADENOSINE ON MYOCARDIAL OXYGEN CONSUMPTION

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1 A 3 min intracoronary infusion of adenosine (50 µg/min) produced a significant decrease in coronary artery perfusion pressure, left ventricular systolic pressure and myocardial O_2 consumption in the isolated supported heart preparation of the dog perfused at a constant coronary blood flow. Heart rate was controlled at 150, 190 or 230 beats/minute.

2 Myocardial contractile force and maximal left ventricular d*p*/d*t* were not changed by adenosine infusion.

3 The absolute decrease in myocardial O_2 consumption was greater at increasing heart rates whereas the decrease in coronary artery perfusion pressure and peak left ventricular systolic pressure were similar.

4 The results suggest that the reduction in myocardial O_2 consumption produced by adenosine is not related to coronary vasodilatation or to a negative chronotropic or inotropic action, but may be due to a functional shunting of blood flow from high O_2 extracting regions of the myocardium to low O_2 extracting ones and/or to important effects on myocardial substrate utilization.

Introduction

Adenosine has been shown to be a potent coronary vasodilator and Berne, Rubio, Dobson & Curnish (1971) have proposed an important role for this compound in coronary vascular autoregulation. However, the effect of adenosine on myocardial O_2 consumption is controversial. Raberger, Kraupp, Stuhlinger, Nell & Chirikdjian (1970) showed a decrease, Bache, Cobb & Greenfield (1973) no change and Afonso & O'Brien (1970) an increase in myocardial O_2 utilization during the administration of adenosine. The present series of experiments was designed to investigate further the role of adenosine on myocardial haemodynamics and O_2 consumption in the isolated supported heart preparation of the dog perfused at a constant coronary blood flow. Adenosine was administered into the coronary artery while the heart rate was held constant at 150, 190 or 230 beats/minute. The results demonstrate that adenosine produces a significant decrease in myocardial O_2 consumption independent of changes in coronary blood flow, heart rate or myocardial contractile force.

Methods

Mongrel dogs weighing between 15 and 20 kg were anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.). The procedure for isolation and perfusion of the recipient heart has been described (Somani, Laddu & Hardman, 1970). Briefly, the heart was isolated *in situ* and the coronary arteries perfused at constant flow with heparinized (5.0 mg/kg) arterial blood from a donor dog. Coronary blood flow was adjusted initially to provide a coronary artery perfusion pressure of 90 mmHg. A thin latex balloon was placed in the left ventricle and inflated with 0.9% w/v NaCl solution (saline) to provide a peak left ventricular systolic pressure of 100 mmHg. Maximal left ventricular d*p*/d*t* was obtained by electronic differentiation of the left ventricular pressure pulse. Myocardial contractile force was determined by a Brodie-Walton strain gauge arch sutured to the left ventricular free wall. Heart rate was controlled by right atrial pacing. Before and during drug infusion simultaneous arterial and coronary venous blood samples were withdrawn and used for measurement of blood pH, $P\mathrm{o}_2$, $P\mathrm{co}_2$, haemoglobin, oxyhaemoglobin and O_2 content.

Myocardial oxygen consumption ($M\dot{V}O_2$)

Direct measurements of arterial and coronary venous blood O_2 content were determined with duplicate 20 µl samples by an electrolytic cell method (Lex-O_2-Con, Lexington Instrument Company). Myocardial O_2 consumption was calculated from the following equation:

$$M\dot{V}O_2 = \frac{(AO_2 - VO_2) \times CBF}{\text{heart weight (g)}} \times 100$$

where $M\dot{V}O_2$ = total myocardial O_2 consumption expressed as ml O_2 utilized/minute per 100 g heart

Table 1 Isolated supported dog heart preparation: summarized haemodynamic and O_2 consumption data ($n=6$)

Parameter	*Control HR (150 beats/min)*	*Adenosine (1)*	*Control HR (190 beats/min)*	*Adenosine (2)*	*Control HR (230 beats/min)*	*Adenosine (3)*
A, O_2 content (vol. %)	20.7 ± 1.0	20.7 ± 1.1	20.7 ± 1.2	20.5 ± 1.1	20.9 ± 1.3	20.8 ± 1.3
V, O_2 content (vol. %)	15.1 ± 1.1	15.5 ± 1.1*	13.5 ± 1.2‡	14.2 ± 1.1†	12.1 ± 1.4‡§	13.2 ± 1.4†
A-V, O_2 content (vol. %)	5.7 ± 0.4	5.2 ± 0.4*	7.2 ± 0.5‡	6.4 ± 0.5†	8.8 ± 0.5‡§	7.7 ± 0.4†
O_2 extraction (AO_2-VO_2/AO_2 %)	27.7 ± 2.3	25.5 ± 2.1*	35.3 ± 2.7‡	31.3 ± 2.4†	43.2 ± 3.9‡§	37.9 ± 3.7†
Myocardial O_2 consumption (ml min^{-1} 100 g^{-1})	4.6 ± 0.5	4.2 ± 0.5*	5.8 ± 0.6‡	5.1 ± 0.6†	7.2 ± 0.7‡§	6.3 ± 0.7†
Heart rate (beats/min)	150	150	190	190	230	230
Contractile force (mm)	22.5 ± 2.5	22.7 ± 2.7	24.4 ± 3.0‡	24.3 ± 3.1	24.2 ± 3.1	23.8 ± 3.2
Coronary artery perfusion pressure (mmHg)	91.0 ± 2.4	78.0 ± 2.9†	87.7 ± 5.0	73.5 ± 6.0†	86.0 ± 7.6‡	75.2 ± 8.0†
Left ventricular systolic pressure (mmHg)	100.0 ± 1.3	90.7 ± 4.4*	100.7 ± 0.7	87.0 ± 3.5*	102.2 ± 1.6	89.5 ± 2.9*
dp/dt (mmHg/s)	875.0 ± 125.0	825.0 ± 125.0	880.0 ± 120.0	875.0 ± 125.0	850.0 ± 50.0	850.0 ± 50.0
Coronary blood flow (ml min^{-1} 100 g^{-1})	82.2 ± 9.2	82.2 ± 9.2	82.2 ± 9.2	82.2 ± 9.2	82.2 ± 9.2	82.2 ± 9.2

Results are mean ± s.e. mean.

* Effect of a 3 min intracoronary infusion (50 µg/min) of adenosine at each heart rate (HR). Significantly different from preceding control by a paired comparison ($P < 0.05$).

† Effect of a 3 min intracoronary infusion (50 µg/min) of adenosine at each heart rate (HR). Significantly different from preceding control by a paired comparison ($P < 0.01$).

‡ Effect of right atrial pacing. Significantly different from control at a heart rate of 150 beats/min by a paired comparison ($P < 0.05$).

§ Effect of right atrial pacing. Significantly different from control at a heart rate of 190 beats/min by a paired comparison ($P < 0.05$).

weight; AO_2 and VO_2 = arterial and venous O_2 content in volumes percent (ml/100 ml); CBF = total coronary blood inflow (ml/minute). Percent extraction of O_2 by the myocardium was calculated from the arterial-venous O_2 content difference divided by the arterial O_2 content.

Experimental design

After isolation the recipient heart was allowed to stabilize for 15 min, then paced at 150 beats/min for 15 min during which time coronary artery perfusion pressure and left ventricular systolic pressure were adjusted to 90 and 100 mmHg, respectively. After control arterial and coronary venous blood samples were withdrawn, adenosine (50 µg/min) was infused into the coronary arterial inflow tubing for 3 minutes. Blood samples were again withdrawn 2–3 min after the start of drug infusion. The preparation was then allowed to return to the control state (30 min) and the process repeated for heart rates of 190 and 230 beats/minute.

Results

After surgery the isolated supported heart usually remained stable. Preparations with ventricular ectopic beats were considered unsuccessful and not used in data analysis. Haemodynamic and metabolic data are summarized in Table 1.

With increasing heart rate (150 to 190 to 230 beats/min) there was a progressive decrease in coronary artery perfusion pressure. Left ventricular systolic pressure was maintained near 100 mmHg. Coronary blood flow controlled by a roller pump remained constant. As heart rate was increased, there was a rate-dependent decrease in coronary venous blood O_2 content and a significant increase in A-V O_2 difference, O_2 extraction, and $M\dot{V}O_2$ (Table 1).

A 3 min intracoronary infusion of adenosine (50 µg/min) produced a significant decrease in A-V O_2 difference, O_2 extraction and $M\dot{V}O_2$ at each heart rate. The decrease in $M\dot{V}O_2$ was paralleled by a significant decrease in coronary artery perfusion pressure and peak left ventricular systolic pressure (Table 1). However, other measured haemodynamic determinants of $M\dot{V}O_2$ (heart rate, coronary blood flow, dp/dt, and contractile force) did not change significantly. Although the decreases in coronary artery perfusion pressure and left ventricular systolic pressure produced by adenosine were of similar magnitude at each heart rate, the decrease in $M\dot{V}O_2$ was greater at the higher rates.

Discussion

Conflicting reports exist concerning the direct cardiac effect of adenosine on $M\dot{V}O_2$. Lammerant, Becsei, Mertens-Strijthagen & de Schryver (1970) and Raberger *et al.* (1970) found a decrease, Hirche (1966) and Weissel, Brugger, Raberger & Kraupp (1974) an increase, and Bache *et al.* (1973) no change in $M\dot{V}O_2$ during infusions of adenosine in dog and cat hearts. The present study clearly demonstrates that adenosine produces a significant decrease in $M\dot{V}O_2$ by a direct cardiac action. The decrease in $M\dot{V}O_2$ occurs independently of any peripheral vascular or reflex changes and occurs independently of alterations in coronary blood flow, heart rate or myocardial contractile force. Moreover, the decrease in coronary artery perfusion pressure and left ventricular systolic pressure is unlikely to be responsible for the decrease in $M\dot{V}O_2$ produced by adenosine since these haemodynamic changes were nearly equivalent at each heart rate whereas the absolute decrease in $M\dot{V}O_2$ was greater with each incremental increase in heart rate.

In addition, large increases in left ventricular systolic pressure (0–225 mmHg) have been previously shown to change $M\dot{V}O_2$ by 0.2–0.5 ml min^{-1} 100 g^{-1} in the isolated supported heart (Somani *et al.*, 1970). Thus, it seems unlikely that a fall of 10–13 mmHg in left ventricular systolic pressure would be responsible for the decrease in $M\dot{V}O_2$ produced by adenosine.

Raberger *et al.* (1970) showed adenosine to have an effect on myocardial substrate utilization. These authors proposed that adenosine decreased $M\dot{V}O_2$ by a lipolytic and glycolytic action on the heart mediated through a stimulation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) production. In contrast, Schauman, Juhran & Dietmann (1970) suggested that adenosine produced a decrease in $M\dot{V}O_2$ via a decrease in myocardial cyclic AMP formation. However, considering the high concentrations of adenosine (> × 10 µM) necessary to inhibit adenylate cyclase *in vitro* (Bär & McKenzie, 1973), it is unlikely that the decrease in $M\dot{V}O_2$ observed in the present study is via this mechanism.

Lammerant *et al.* (1970) suggested that the reduction in $M\dot{V}O_2$ produced by adenosine may be due to a functional shunting or redistribution of blood flow from a high O_2 extracting region (subendocardium) to a low O_2 extracting region (subepicardium) of the myocardium. It is possible that adenosine opens up vessels in the myocardium which do not participate in cellular gas exchange and thereby produces an apparent reduction in the calculated $M\dot{V}O_2$. Because regional myocardial blood flow was not measured in the present series of experiments, this mechanism could not be evaluated. Further work is needed to establish whether adenosine reduces $M\dot{V}O_2$ by producing a redistribution of blood flow within the myocardium or by a direct metabolic action.

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DECREASED PHENYLETHANOLAMINE-*N*-METHYLTRANSFERASE AND CATECHOL-*O*-METHYLTRANSFERASE ACTIVITY IN RABBIT ADRENAL GLANDS DURING PREGNANCY

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1 Phenylethanolamine-*N*-methyltransferase (PNMT) activity in adrenal gland was lower than the control values at day 24 of rabbit pregnancy and fell throughout the gestational period.

2 Catechol-*O*-methyltransferase (COMT) activity during pregnancy and parturition followed a similar pattern to PNMT.

3 Adrenaline content of the adrenal gland declined significantly during the last few days of pregnancy but at parturition showed a tendency to return towards normal.

4 The biochemical mechanisms which result in a decline in PNMT and COMT activities and adrenaline concentration appear to be the consequence of modifications in endocrine gland secretion during pregnancy.

Introduction

The role of adrenergic neurotransmission in the maintenance of pregnancy has been discussed by many investigators (Fuchs, 1971). Animal experiments provide evidence that the monoamine content of peripheral and central nervous systems is altered during pregnancy (Kopin & Wurtman, 1963; Sjöberg, 1968). The binding, uptake and metabolism of radioactively labelled amines by the tissues of pregnant animals differ from those of non-pregnant female animals (Wurtman, Axelrod & Potter, 1964; Parvez, Parvez & Youdim, 1975). Our previous studies have shown that the processes of monoamine regulation have an important link with the termination of pregnancy (Parvez, Gripois & Parvez, 1973a; Parvez, Parvez & Gripois, 1973b) in the rabbit, whilst pregnant women have a significantly elevated neurotransmitter monoamine content in the blood close to onset of labour (Zuspan, 1970; Zuspan & Zuspan, 1973). The present study was designed to investigate the influence of pregnancy and parturition upon monoamine biosynthetic and catabolic enzymes in the rabbit adrenal gland.

Methods

Variations in rates of adrenaline biosynthesis and catabolism were measured by determining the activities of the enzymes phenylethanolamine-*N*-methyltransferase (PNMT) and catechol-*O*-methyltransferase (COMT). Adrenal monoamine content was also determined at those times during pregnancy when activities of the enzymes PNMT and COMT were likely to have changed. Albino rabbits of New Zealand strain were used. They were impregnated in the laboratory and kept in separate cages in an animal house at a constant temperature of 20°C with exposure to natural light and darkness. Under these conditions, parturition occurred during days 31–32 post coitum. At specified days of pregnancy, the animals were killed by neck fracture and the adrenals were rapidly excised.

*Assay of phenylethanolamine-*N*-methyltransferase*

The right adrenal was homogenized in 2 ml ice cold 0.9% (w/v) KCl and the homogenate centrifuged for 30 min at 50,000 *g* at 4°C. The supernatant was used as the source of the enzyme. The incubation mixture consisted of 0.2 ml phosphate buffer (0.5 M, pH 7.9), 20 µl of normetadrenaline solution (4.5 mg/ml), 0.2 ml enzyme preparation and 0.1 ml of *S*-adenosyl-methionine-[^{14}C-methyl] (20 nmol). The mixture was incubated for 1 h at 37°C. The reaction was stopped by addition of 0.5 ml borate buffer (0.5 M, pH 10). This was followed by addition of 4 ml toluene plus isoamyl alcohol (3:2 ratio). The tubes were shaken mechanically for 20 min and centrifuged for 5 min at

5000 *g*; 3 ml of the organic phase was transferred to a scintillation bottle containing 10 ml Bray's phosphor. The ezyme activity is expressed either as ct/min per whole adrenal gland or as ct min^{-1} g^{-1} of adrenal gland, since the extracted ct/min showed direct proportionality to the amount of enzyme preparation used (Parvez & Parvez, 1973).

Assay of catechol-O-methyltransferase

COMT was assayed using *S*-adenosylmethionine-[^{14}C-methyl] (Parvez & Parvez, 1973) as methyl donor. The enzyme preparation was the same as for PNMT. The incubation mixture consisted of 0.2 ml phosphate buffer (0.5 M, pH 7.9), 20 µl $MgCl_2$, 20 µl (–)-adrenaline (0.5 µM), 0.1 ml *S*-adenosylmethionine-[^{14}C-methyl] (20 nmol) and 0.2 ml enzyme preparation. The rest of the procedure was the same as that used for the assay of PNMT. The enzyme activity is expressed as described above.

Adrenal monoamine determination

The left adrenal gland was freed from fat, weighed and homogenized in 2 ml 4% (w/v) trichloroacetic acid in an all glass homogenizer. After centrifugation at 50,000 *g*, the supernatant was assayed for adrenaline by the method of Euler & Lishajko (1961): the trihydroxindole reaction was employed for spectrofluorometric determination of adrenaline.

All results are shown with standard errors of the mean. The significance of differences between means was calculated using Fisher's *t* test.

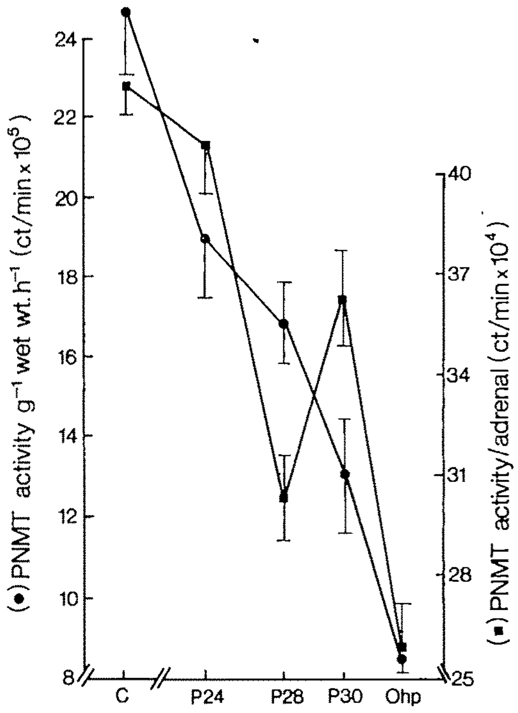

Figure 1 Activity of phenylethanolamine-*N*-methyltransferase (PNMT) in the adrenal glands of non-pregnant, (C) and pregnant rabbits (P24, 28 and 30 days post coitum) and rabbits at parturition (OhP, zero hour after birth when all the foetuses were born). The results are expressed as mean of 6 to 12 animals. Vertical lines show s.e. mean. Significance of differences between groups: C *vs* OhP, $P<0.001$ (ct min^{-1} g^{-1} and ct min^{-1} per adrenal; 30 days post coitum *vs* OhP, $P<0.01$ (ct min^{-1} g^{-1} and ct min^{-1} per adrenal).

Results

Figure 1 shows variations in PNMT activity during the course of pregnancy. Activity was lower than control values at day 24 of pregnancy and was observed to fall throughout the gestational period whether the results are expressed as ct min^{-1} g^{-1} wet wt or as ct/min per adrenal. Enzyme activity, when expressed in ct min^{-1} g^{-1} wet wt, showed a constant decline to the end of parturition. At parturition, PNMT activity (ct min^{-1} g^{-1}) was one third of control value. PNMT activity showed a significant increase on day 30 post coitum when compared with the value on day 28 when the results were expressed as ct/min per adrenal. COMT activity during pregnancy and parturition (Figure 2) showed a

Table 1 Adrenaline content of rabbit adrenal glands during pregnancy

Animals	*Adrenaline (µg/pair)*	*Adrenaline (µg/g wet wt.)*	*% of Control (µg/g)*	*(µg/pair)*
Control	65 ± 10	600 ± 50		
28 days post coitum	40 ± 15	410 ± 40	60%	68%
30 days post coitum	20 ± 5	320 ± 20	30%	53%
0 hour parturition	45 ± 8	410 ± 10	69%	68%

The results are expressed as mean (± s.e. mean of 10 to 15 animals). Significance of differences between groups: control vs 30 days post coitum, $P<0.005$ (µg/g), $P<0.001$ (µg/pair) and 30 days post coitum vs 0 hour parturition, $P<0.002$ (µg/g) and $P<0.002$ (µg/pair).

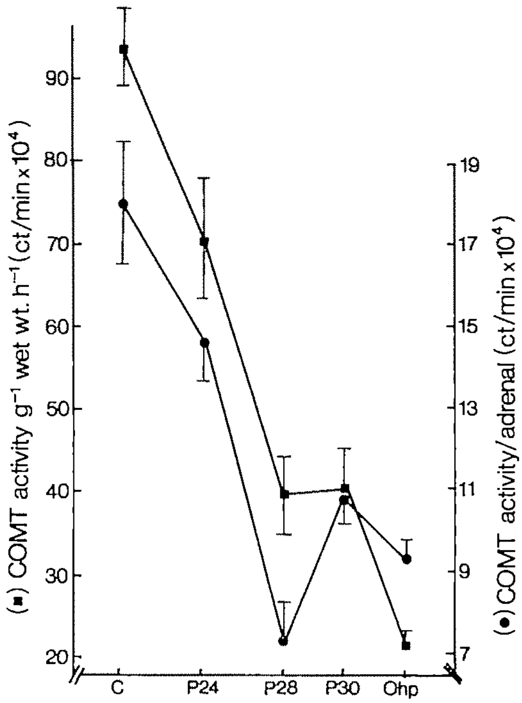

Figure 2 Activity of the enzyme catechol-*O*-methyltransferase (COMT) in the adrenal glands of non-pregnant (C) and pregnant rabbits (P24, 28 and 30 days post coitum) and rabbits at parturition (OhP, zero hour after birth when all the foetuses were born). The results are expressed as mean of 6 to 12 animals. Vertical lines show s.e. mean. Significance of differences between groups: C *vs* 28 days post coitum, $P<0.001$ (ct min^{-1} g^{-1} and ct/min per adrenal) and 30 days post coitum *vs* Ohp $P<0.002$ (ct min^{-1} g^{-1}).

similar pattern to that of PNMT. At 28 days post coitum, activity was lower than control values but higher than at 30 days post coitum when the results were expressed as ct/min per adrenal gland. Adrenaline content of adrenal gland declined significantly during the last few days of pregnancy but at parturition there was a tendency to return towards normal values (Table 1).

Discussion

From the present experiments, it is evident that the rate of adrenaline formation from noradrenaline declines throughout the duration of pregnancy, the fall being most marked at time of parturition. The decline in adrenaline content of the adrenal gland during late pregnancy is in keeping with the finding that PNMT activity is directly affected by the state of gestation. If, during pregnancy, synthesis and catabolism are both functioning at a lower rate, there should be no significant change in monoamine content of adrenal glands. However, the observed decline in adrenaline concentrations in the adrenal gland suggests an accelerated release of this amine into the blood. This suggestion is strengthened by the studies of Zuspan & Zuspan (1973) who reported an increased output of catecholamines during late pregnancy and parturition, a finding confirmed by others (Parvez *et al.*, 1973a). A later study (Parvez *et al.*, 1973b) showed that during the last part of pregnancy (30 days post coitum) and parturition in rabbits, there was a 3–4 fold increase in plasma adrenaline and noradrenaline content. Furthermore, a few hours before parturition noradrenaline dropped to its minimum level whereas adrenaline remained higher, which points to some modification of PNMT activity at parturition.

The biochemical mechanisms responsible for the decline in PNMT activity appear to be connected with modifications in endocrine gland secretion. It has been reported that corticoids induce the synthesis of PNMT (Wurtman & Axelrod, 1966; Margolis, Roffi & Jost, 1966). Subsequent study of the response of extra-adrenal chromaffin tissue to corticosterone in organ culture (Coupland & MacDougall, 1966; Coupland, 1968; Barber, Coupland & Crowe, 1971) demonstrated that corticoids control the formation of adrenaline in cells which would otherwise store noradrenaline and that this effect is accompanied by induction of PNMT. Recently Mulay, Giannopoulos & Solomon (1973) have shown that the concentration of corticosterone in rabbit adrenal gland during pregnancy falls to one third of the value found in non-pregnant rabbits. The fall occurs 24 days post coitum and persists until parturition. These results may explain the low PNMT activity observed in our studies for at this time, lower concentrations of corticosterone are present to stimulate PNMT activity. A similar explanation may conceivably be applied to the lowered COMT activity which is also a methyltransferase enzyme. An analysis of *in vivo* and *in vitro* effects of sex steroid hormones on monoamine catabolism has been made recently by Youdim, Holzbauer & Woods (1974) and others (Parvez *et al.*, 1975) in non-pregnant and pregnant rats. Oestrogens and progesterone alter COMT activity (Wurtman & Axelrod, 1966; Parvez *et al.*, 1975). Blood concentrations of oestrogens and progesterone change considerably during late pregnancy (Shaikh, 1971; Weist, 1970) and both hormones have been shown to exert an important influence on monoamine catabolism (Holzbauer & Youdim, 1973; Southgate, 1972) with progesterone acting as a stimulator and oestrogens as inhibitors.

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THE INTERACTION BETWEEN ANTHELMINTIC DRUGS AND HISTAMINE IN *Ascaris suum*

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1 Piperazine reduced the histamine content of *Ascaris suum*, yet it greatly increased the uptake of histamine from the surrounding medium, the neuromuscular structures of the nematode preferentially increasing in amount.

2 Bephenium reduced the histamine content of *Ascaris* and the uptake of histamine from the surrounding medium. However, the relative amount in the neuromuscular structures increased.

3 The flaccid paralysing action of piperazine may thus involve increased histamine absorption whereas the spastic paralysing action of bephenium may be independent of histamine.

Introduction

The spindle-shaped, longitudinal muscles of the body wall of *Ascaris suum* consist of cells which are divided into two portions, contractile and non-contractile. Processes pass from the non-contractile portions to the lateral nerves and the circumpharyngeal nerve ganglion where they form a complex syncytium. Two types of junction are apparent at this level, one between muscle arms and one between muscle arms and nerve cords (de Bell, 1965). When the nerve cords are stimulated, both inhibitory and excitatory effects on the muscle can be identified. The natural excitatory transmitter may be acetylcholine (Gerschenfeld, 1973) but the inhibitory transmitter is still not known. Histamine has been proposed as an inhibitor for it occurs in the tissues of *Ascaris* and is concentrated in the neuromuscular structures (Miyagawi, 1961; Phillips, Sturman & West, 1975*a*). Furthermore, histamine inhibits acetylcholine-induced contractures of muscle preparations of *Ascaris*.

Piperazine, an anthelmintic agent used in the treatment of Ascariasis, causes flaccid paralysis of the parasites and may be acting via an inhibitory system, either by direct stimulation or by interference with the natural inhibitory transmitter. The results of preliminary experiments (Phillips, Sturman & West, 1975*b*), showed that piperazine reduced the histamine content of *Ascaris suum* but it greatly increased the uptake of histamine from the incubation fluid. In fact, the parasites always exhibited a greater degree of paralysis when they were treated with piperazine and histamine together than with either compound alone. As histamine is a natural constituent of the intestines of the host, it was decided to investigate further the interaction between histamine and piperazine in *Ascaris* and compare it with that between histamine and bephenium, an anthelmintic drug producing spastic paralysis.

Methods

Mature, female *Ascaris suum* were collected from the intestines of freshly slaughtered pigs and transported to the laboratory in modified Baldwin-Ringer solution (Baldwin & Moyle, 1947) containing antibiotics (penicillin G, 10,000 i.u./ml; streptomycin, 1.5 mg/ml; tetracycline, 0.5 mg/ml; sulphanilamide, 1.2 mg/ml) and an antimycotic (Nystatin, 1.5 mg/ml) to prevent contaminating micro-organisms contributing to the histamine content of the *Ascaris*. Groups of five parasites were incubated in 1 litre quantities of the Ringer solution at 38°C for 24 h, the medium being changed twice during this period. Drugs were added to the Ringer solution at the start of the incubation. The *Ascaris* were then washed 6 times with drug-free Ringer solution, dried, stunned, weighed and the histamine extracted into *n*-butanol prior to assay fluorimetrically (Shore, Burkhalter & Cohn, 1959) and biologically (Phillips *et al.*, 1975*a*). In other experiments, whole *Ascaris*, after incubation and stunning, were carefully dissected into head and body wall, intestines, reproductive tract and perienteric fluid before being weighed, extracted and assayed.

In a few experiments with the anthelmintic drugs, [ring ^{14}C]-histamine was used to study uptake and distribution of histamine after incubation.

Histidine decarboxylase activity

This was determined by a modification of the method of Telford & West (1961). The incubation mixtures consisted of homogenized *Ascaris* (4.9 ml of 5 ml/g extract in 0.9% w/v NaCl solution), phosphate buffer (pH 6.0, 4.9 ml) or phosphate buffer containing piperazine or bephenium (final concentration 1 mg/ml), aminoguanidine (0.1 ml of 10 mg/ml) and histidine (0.1 ml of 100 mg/ml). The solutions were thoroughly mixed and incubated with shaking for 3 h at 38°C. Then, 4 ml aliquots were added to 4 ml 0.4 M perchloric acid and taken through *n*-butanol extraction for fluorimetric as well as biological assay for histamine. Blanks contained phosphate buffer instead of histidine.

Diamine oxidase activity

The incubation mixtures consisted of hog kidney diamine oxidase (0.1 ml of 0.5 i.u./ml), phosphate buffer (pH 6.3, 4.8 ml) or phosphate buffer containing piperazine or bephenium (final concentration 1 mg/ml) and histamine (0.1 ml of 5 mg/ml). The solutions were thoroughly mixed and incubated for 1 h at 38°C. The reaction was then stopped by the careful addition of 0.1M HCl and the histamine was assayed fluorimetrically and biologically. Suitable blanks were prepared in which diamine oxidase, histamine or the anthelmintics were omitted.

Drugs

The drugs used were as follows: histamine acid phosphate (Sigma), histidine hydrochloride (Sigma), piperazine hexahydrate (Sigma), bephenium hydroxynapthoate (The Wellcome Foundation Ltd.), diamine oxidase (Sigma) and [ring-2-^{14}C]-histamine dihydrochloride (Radiochemical Centre, Amersham).

Results

Effect of anthelmintics on histamine content

The mean (± s.e. mean) histamine content of 10 control *Ascaris* after incubation for 24 h at 38°C was 93 ± 6 mg/gram. Both piperazine and bephenium significantly reduced the histamine content of *Ascaris* by 19% and 28% respectively (Table 1). Some inhibition of movement of the nematodes was noted when each drug was present.

Effect of anthelmintics on histamine uptake

In the presence of histamine (10 µg/ml), the histamine content increased about three-fold (Table 2), but, when piperazine was included in the incubation fluid together with the histamine, a seven-fold increase in content was obtained. On the other hand, bephenium greatly reduced histamine uptake. These changes in the histamine content of *Ascaris* in the presence of piperazine and bephenium are illustrated in Figure 1. Similar results were obtained when the uptake of radioactive histamine was studied.

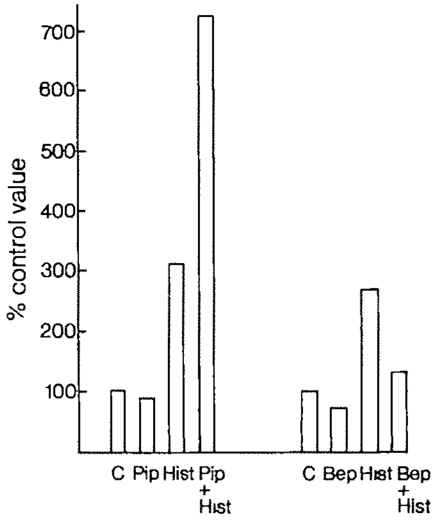

Figure 1 Changes in the histamine content of *Ascaris suum* after incubation with piperazine or bephenium in the absence or presence of histamine. Pip = piperazine 1 mg/ml: Hist = histamine 10 µg/ml: Bep = bephenium 1 mg/ml: C = control value without drugs. Histamine contents are percentages of control values taken as 100%. Note that piperazine increases uptake of histamine whereas bephenium decreases uptake.

Distribution of histamine after incubation

Table 3 shows the relative histamine content of parts of the nematode after incubation with Ringer alone. More than half of the total histamine was found to be present in the head and body wall (where the muscle and nerve processes are located). When histamine was included in the incubation mixture, this percentage

Table 1 The histamine content (ng/g) of *Ascaris suum* after incubation at 38°C for 24 h with piperazine or bephenium

Anthelmintic	*Piperazine (1 mg/ml)*	*Bephenium (1 mg/ml)*
Absent	82 ± 5	104 ± 7
Present	67 ± 6	75 ± 5
% Decrease	19	28
Significance	$P < 0.05$	$P < 0.05$

Histamine contents are expressed as mean ± s.e. mean of 5 experiments.

figure (55%) did not change although the total histamine increased some three-fold, but in the presence of piperazine or bephenium relatively more was found in this part of the nematode. For example, 73% of the total histamine was in the head and body wall when piperazine and histamine were in the incubation mixture, whereas with bephenium it was 63% of the total.

Histidine decarboxylase

The activity of this enzyme from *Ascaris* was inhibited 55% by piperazine (1 mg/ml) yet bephenium (1 mg/ml) had no effect.

Diamine oxidase

Piperazine (1 mg/ml) had no effect on the activity of this enzyme yet bephenium (1 mg/ml) stimulated the inactivating enzyme by over 30%.

Discussion

The results of the present experiments show that *Ascaris* readily take up histamine. In the presence of piperazine (a drug which by itself reduces the histamine content), the uptake of histamine is further increased. The relative amount of histamine in the neuromuscular structures (that is, head and body wall) increased and it was noted that under these conditions the paralysing action of piperazine was augmented. On the other hand, in the presence of bephenium (a drug which also reduces the histamine content of the worm), the uptake of histamine is decreased. The relative amount of histamine in the neuromuscular structures increased although no change was found in the spastic paralysing action of bephenium.

Piperazine may owe part of its paralyzing action on *Ascaris suum* to its ability to increase the uptake of histamine from the surrounding medium. The pig's intestinal fluid contains about 10 μg/ml histamine (Phillips *et al.*, 1975*b*) and so *in vivo* the uptake may well occur. Histamine is concentrated in the neuromuscular structures and it produces flaccid paralysis of the parasite. However, this effect can only be contributory to the action as piperazine itself produces paralysis and inhibits histidine decarboxylase, the specific enzyme involved in the formation of histamine within the parasite. Such an inhibition may explain the reduction in histamine content when piperazine was used alone.

Table 2 The histamine content (ng/g) of *Ascaris suum* after incubation with histamine or with piperazine or bephenium in the presence of histamine

Anthelmintic	*Histamine (10 μg/ml)*	*Piperazine (1 mg/ml)*	*Bephenium (1 mg/ml)*
Absent	Absent	104 ± 9	137 ± 9
Absent	Present	335 ± 43	366 ± 28
Present	Present	778 ± 133	187 ± 15
% Change		191% increase	78% decrease
Significance		$P<0.05$	$P<0.05$

Histamine contents are expressed as mean ± s.e. mean of 5 experiments.

Table 3 The relative percentage distribution of histamine in the tissue of *Ascaris suum* after incubation alone, with histamine, or with piperazine or bephenium in the presence of histamine

Tissue	*Control*	*Histamine*	*Piperazine and histamine*	*Bephenium and histamine*
Head and body wall	55	54	73	63
Reproductive tract	19	20	14	16
Intestines	15	8	7	8
Perienteric fluid	11	18	6	13

Results are the mean values of 10 experiments.

Bephenium, which produces spastic paralysis of *Ascaris suum*, also lowers the histamine content of the worm but apparently by stimulating its metabolism rather than by inhibiting its synthesis. It also reduces the uptake of histamine from the surrounding medium. However, as with piperazine, these interactions with histamine are probably secondary to a more major mechanism of action of bephenium in producing paralysis of the parasites. The histamine content of *Ascaris* following incubation with histamine and bephenium is not significantly different from that of fully mobile parasites taken straight from the pig's intestine.

Other workers (for example, Jones, Rothwell, Dineen & Griffiths, 1974) have found that during the course of infection of guinea-pigs with another gastrointestinal nematode, *Trichostrongylus colubriformis*, histamine levels in the small intestine increase about three-fold. Furthermore, guinea-pigs immunized by previous infection and challenged with *T. colubriformis* show an additional increase in intestinal histamine levels just prior to worm expulsion while the infusion of histamine together with 5-hydroxytryptamine (5-HT) into the small intestines of animals during the 4th larval stage of a primary infection leads to significant worm expulsion (Rothwell, Pritchard & Love, 1974). Histamine may exert an action on either the parasite or host or on both to assist expulsion of the worms. If an action on the parasite by histamine is considered, then it could possibly be a stimulation of an inhibitory nervous system, as postulated for *Ascaris suum*. Any treatment which increases histamine uptake would theoretically promote worm expulsion. It would therefore be of interest to see if piperazine increases the uptake of histamine by *T. colubriformis*. Drugs which inhibit the action of histamine are known also to inhibit the expulsion of *T. colubriformis* by infected guinea-pigs.

5-HT levels in the intestines also rise during infection with intestinal nematodes and this system may be implicated in the mechanism of action of anthelmintics as peristalsis is controlled, in part, by the formation and release of 5-HT. Further work is now in progress studying the interaction between 5-HT and anthelmintics.

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The toxicology of the tetrachloroethanes

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1,1,2,2-Tetrachloroethane (sym-TC1E) is a solvent with wide industrial application whereas the unsymmetrical isomer, 1,1,2-tetrachloroethane (unsym-TC1E) is only occasionally used in the laboratory. The latter isomer has been shown to cause a prolongation of hexobarbitone-induced sleeping time in rats and appears to be a protein synthesis inhibitor (El Tayeb, 1974). The hepatotoxicity of the tetrachloroethanes was studied at the subcellular level by measurement of succinate dehydrogenase, acid phosphatase and glucose-6-phosphatase activity and DNA content. These enzymes and DNA were used as indices of the functions of the mitochondria, lysosomes, endoplasmic reticulum and nucleus respectively. Concomitant haematological studies were also carried out and changes in white and red cell counts and lipid concentrations were noted.

Symmetrical- and unsym-TC1E in a dose range of 100 to 800 µmol/kg body weight were administered by intraperitoneal injection daily for 7 days to male rats. With these dose regimens no toxic symptoms were observed *in vivo*, however, there was a significant ($P \leqslant 0.05$) increase in the activities of all enzymes studied which was accompanied by a decrease in DNA content. At the same time there was an increase in the white cell count and decreases in the red cell count and cholesterol content. All changes observed both in the liver and blood were dose and isomer dependent but showed no predictable pattern. These results are discussed in relation to proposed mechanisms for the toxicity of aliphatic organohalogen compounds.

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Absorption and metabolism of steroids administered intratracheally to rat isolated lungs

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A number of endogenous and exogenous substances are metabolized on passage through the pulmonary circulation (for refs. see Bakhle & Vane, 1974). Steroids are metabolized by homogenates of lung from several species (Sowell, Hagen & Troop, 1971; Hartiala, Nienstedt & Hartiala, 1973; Hartiala, 1974) and recently the metabolism of two steroids on transit through the pulmonary circulation of isolated lungs has been demonstrated. [^{3}H]-Cortisol was detected in the venous effluent of rat lungs perfused with Krebs solution containing [^{3}H]-cortisone (Nicholas & Kim, 1975). In the same tissue [^{14}C]-testosterone was taken up and metabolized avidly when the perfusate was protein-free (45% of the radioactivity in the lung was metabolite) but less (29%) when diluted rat blood was used as perfusate (Hartiala, Uotila & Nienstedt, 1976). With the introduction of steroids in aerosol form into clinical practice we decided to study the uptake and metabolism of two steroids, testosterone and beclomethasone diproprionate, administered intratracheally to rat isolated lungs.

Male rat lungs were perfused via the pulmonary artery with fresh rat blood diluted with Tyrode solution (haematocrit 0.28) at a flow rate of 10 ml/min and ventilated with room air. In 'single pass' experiments the perfusate was not recirculated and the effluent was collected in two portions, 0–2 min and 2–5.5 minutes. Analysis of effluent and lung samples was carried out as described previously (Nienstedt, 1967; Nienstedt & Hartiala, 1969). The steroid (4-[^{14}C]-testosterone, 57.7 nCi/nmol, New England Nuclear; 16,16α-[^{3}H]-beclomethasone diproprionate, 55 nCi/nmol) was instilled into the airways and the perfusion started. With testosterone (0.4 nmol in 0.1 ml of isotonic saline), 2 min after instillation, 40% of the radioactivity had appeared in the perfusate, the majority (89%) as unchanged testosterone. For comparison, cortisol and dexamethasone similarly applied to rat lungs are absorbed with half-lives of 1.0 and 1.7 min respectively (Burton & Schanker, 1974). In our experiments, analysis of the radioactivity left in the lung at 2 min showed only 33% to be unchanged testosterone. No conjugated metabolites of testosterone were found in either effluent or lung.

With beclomethasone dipropionate (10–16 nmol in 0.1 ml isotonic saline), the initial rate of absorption of radioactivity from the lungs to the perfusion medium proceeded with a half-life of 15–20 minutes. These results are comparable with those of Martin, Harrison & Tanner (1975). Analysis of the radioactivity remaining in the lung at 2 min showed that about half of the original amount was present as unchanged steroid. The major metabolite (about 30%) had the same mobility as beclomethasone monopropionate on thin layer chromatography. Five min after instillation, the proportion of unchanged steroid had dropped to one third with a considerable increase in polar metabolites other than the monopropionate. Further identification of the polar metabolites of beclomethasone was made difficult by the lack of reference compounds.

From these results we conclude that the slow absorption rate from the trachea and the extensive intra-pulmonary metabolism of beclomethasone may account for the low adrenal suppression observed with beclomethasone aerosol.

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Biphasic changes in motor behaviour following morphine injection into the nucleus accumbens

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Morphine and morphine-like agents are able to initiate changes in motor behaviour which are characteristic both of dopamine antagonism (catalepsy) and dopamine stimulation (stereotyped behaviour and hyperactivity). Extensive electrolesion studies have given some indication of the dopamine-containing areas involved with mediating catalepsy (the amygdala) and stereotypy (the striatum) (Costall & Naylor, 1973), but the involvement of the different areas with the hyperactivity response to morphine-like agents has not been widely investigated. One area which would appear a suitable substrate for this response is the nucleus accumbens. Dopamine and related agents injected directly into the nucleus accumbens cause hyperactivity which may be modulated by 5-hydroxytryptamine (Costall, Marsden, Naylor & Pycock, 1976). The ability of morphine to increase dopamine function, at least as indicated by stereotypy experiments, is also modulated by 5-hydroxytryptamine (Costall & Naylor, 1975).

Therefore, we investigated the effect of morphine on motor behaviour after bilateral injection into the nucleus accumbens of rats. The technique for intracerebral injection was as described by Costall & Naylor (1975).

Initial studies showed that morphine (1–100 µg) administered bilaterally into the nucleus accumbens, in the absence of any pretreatment, caused biphasic changes in motor activity: a period of catalepsy (catatonia), followed by the development of hyperactivity and stereotyped biting. Hyperactivity was measured in perspex observation cages fitted with photocells, whilst, in separate experiments, catalepsy was measured as the time an animal would maintain an abnormal imposed position with both front limbs extended over a 10 cm. high bar. For example, for morphine (50 µg) the phase of immobility developed within 7 min, persisted for 220 min, immediately followed by hyperactivity, which lasted for a further 200 minutes.

The phase of catalepsy (morphine, 12.5 µg) was inhibited in a dose-dependent manner by nalorphine (2.5–10.0 mg/kg s.c.) and cyproheptadine (0.625–5.0 mg/kg i.p.). Piperoxan (10 mg/kg i.p.) and atropine (2.5 mg/kg i.p.) were ineffective, whilst cataleptic doses of haloperidol enhanced the morphine-induced catalepsy.

Nalorphine (10–40 mg/kg s.c.), aceperone (1.25–20.0 mg/kg i.p.) and piperoxan (20 mg/kg i.p.) reduced the intensity of the hyperactivity/biting phase (50 µg morphine) in a dose-dependent manner, however, cyproheptadine (5 mg/kg i.p.) and fluphenazine (1.25 mg/kg i.p.) were only effective in doses causing motor depression in normal animals. Atropine (5 mg/kg i.p.) and propranolol (10 mg/kg i.p.) failed to attenuate the hyperactivity.

It is concluded that the integrity of the nucleus accumbens is important for the actions of morphine, both to cause immobility and to increase activity. However, whilst dopamine and 5-hydroxytryptamine function would appear important for the production of catalepsy, noradrenergic mechanisms may be more important for the expression of hyperactivity.

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Noradrenergic influences on dopamine-dependent behaviour in rats

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It is difficult to assess the precise neurotransmitter roles of noradrenaline in the brain. It has been suggested that noradrenaline may play a modulator role in the control of motor activity (Andén & Strömbom, 1974), and its action may be facilitation of the nigro-neostriatal dopaminergic system of the brain (Pycock, Donaldson & Marsden, 1975). However, it has been demonstrated that a noradrenergic component is apparently not important for the production of amphetamine-induced locomotor activity (Roberts, Zis & Fibiger, 1975) or stereotyped behaviour (Creese & Iversen, 1975) in rats, although an intact dopamine system is necessary (Creese & Iversen, 1974). Work described here has utilized two animal models with lowered cerebral noradrenaline levels to study the effect on behaviour in rats characteristically observed after dopamine receptor stimulation and dopamine receptor blockade.

Depletion of cerebral noradrenaline was achieved in two separate groups of rats by (i) injection of 6-hydroxydopamine at birth, and (ii) bilateral electrolesions placed in the region of the locus coeruleus in adult rats. When both groups, plus an additional litter-mate control group, had reached adult stage, the intensity of catalepsy induced by the dopamine receptor blocking agent haloperidol (range 0.1–2 mg/kg) was observed. A month later, the stereotyped behaviour induced by both directly and indirectly acting dopamine receptor agonists (apomorphine, 0.1–5 mg/kg s.c., and amphetamine, 0.1–10 mg/kg i.p.) was compared in the 3 animal groups. After a further month, animals were killed for determination of forebrain monoamine levels.

Both 6-hydroxydopamine at birth and bilateral locus coeruleus lesions specifically harmed the dorsal noradrenergic bundle innervation of forebrain structures. Dopamine and 5-hydroxytryptamine levels were not changed. Cortical noradrenaline levels fell to between 40–50% of control levels ($P < 0.001$) for both types of lesions.

Cerebral noradrenaline depletion had no effect on stereotyped behaviour induced by either apomorphine or amphetamine, but it did significantly enhance both the time to onset and intensity of catalepsy induced by haloperidol at all doses used.

It is difficult to comment on the mechanism by which lowered central noradrenaline levels modifies catalepsy, due to apparent dopamine receptor blockade, but does not influence stereotypy resulting from dopamine receptor stimulation. It is possible that two different populations of dopamine receptor are responsible for the two types of behaviour, and that each is modified by different noradrenergic mechanisms.

(C.P. is a Fellow of the Parkinson's Disease Society.)

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Paradoxical aversive property of dexamphetamine

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The intravenous self-administration of dexamphetamine shows that it can serve as a reinforcer in rats (Pickens & Thompson, 1971) but a paradoxical, aversive property has been observed with oral self-administration (Le Magnen, 1969; Stolerman, Kumar & Steinberg, 1971). Rats have also been shown to reject distinctively flavoured solutions when their previous consumption was followed by intraperitoneal dexamphetamine (Cappell & LeBlanc, 1971), and a modified procedure involving discrimination between two flavours has been used to analyse further the aversive action of the drug.